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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The goal of this work is to find new treatment strategies for poisoning by nerve agents. A gene therapy protocol capable of delivering human acetylcholinesterase into dividing as well as nondividing cells is being developed, using adeno-associated virus to transfer the gene.

The idea that an organophosphate hydrolase enzyme in the right location will provide protection against organophosphorus agents is being tested in a mouse. A gene targeted mouse that has the human butyrylcholinesterase G117H gene in place of the acetylcholinesterase gene is being made. The G117H enzyme is of special interest because it not only hydrolyzes acetylcholine but also hydrolyzes organophosphorus toxicants.

Another type of mouse, one that contains random insertions of the human butyrylcholinesterase G117H gene, has been made. This transgenic mouse contains multiple copies of the human gene in its chromosomes. The plan is to make a stable transgenic mouse line and then test it for resistance to the toxicity of organophosphorus agents.

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gene therapy, butyrylcholinesterase, nerve agent, organophosphorus toxicant, acetylcholinesterase

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Abbreviations

AAV	adeno-associated virus
AChE	acetylcholinesterase enzyme
ACHE	acetylcholinesterase gene
BChE	butyrylcholinesterase enzyme
BCHE	butyrylcholinesterase gene
DTNB	dithiobisnitrobenzoic acid
Elisa	enzyme linked immunosorbent assay
FLAG	8 amino acid peptide DYKDDDDK
G117H	human butyrylcholinesterase containing Histidine 117 in place of Glycine 117
MEPQ	7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide
OP	organophosphorus toxicant
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
VX	O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate; nerve agent

Introduction

The goal of this work is to protect the soldier from the toxicity of nerve agents. The research plan involves finding new ways to deliver protective cholinesterase enzymes into tissues. Gene therapy and protein therapy protocols are being developed.

Test animals are being made to test the idea that an organophosphate hydrolase enzyme in the right location will provide protection against organophosphorus toxins. The G117H knockin mouse and the G117H transgenic mouse will give information on the level of protection that can be achieved by introducing the gene for an organophosphorus hydrolase.

In this first year of the project, we have made progress on tasks 1, 2, 3, and 4.

Task 1. *Various types of AChE enzyme will be injected into mice for the purpose of determining whether AChE enters the brain and other tissues.*
1.1 Tetramers of fetal bovine AChE will be injected intraperitoneally into AChE-/- mice.

Task 1.1

Abstract

Background. Pretreatment with AChE or BChE protects monkeys against a maximum of 5 LD_{50} doses of soman. Why is there a ceiling for protection? Since one mole of scavenger detoxifies one mole of nerve agent, the level of protection should theoretically be limited only by the amount of scavenger available.

Hypothesis. Exogenously administered native AChE does not enter certain compartments and therefore cannot protect against toxins that escape from the circulation.

Method. AChE -/- mice were injected with bovine AChE. AChE activity was measured in brain and blood. Knockout mice were observed for changes in tremor, temperature, righting reflex, and weight.

Result. Bovine AChE did not enter the brain. The phenotype of AChE -/- mice did not improve following AChE injection, which means AChE did not enter nerve synapses.

Conclusion. AChE and BChE scavengers can protect only against nerve agents that stay in the same compartment as AChE and BChE. The ceiling of protection is explained by escape of nerve agent into tissues inaccessible to detoxication by exogenously administered AChE and BChE.

Introduction

Why is there a ceiling for protection against nerve agent toxicity? Pretreatment of rhesus monkeys with fetal bovine serum AChE or human serum BChE protects against 5 LD_{50} of soman challenge, but not against higher doses (Wolfe et al., 1992). Table 1.1 shows that protection was achieved against a maximum of 1 to 8 LD_{50} doses of sarin, soman, VX or MEPQ, but not against higher doses. Pretreatment with higher amounts of cholinesterase did not allow animals to survive a higher dose of nerve agent. A possible explanation is that high doses of nerve agent escape into brain and other compartments where AChE and BChE cannot follow. The present work tests the idea that i.p. injected AChE cannot reach certain vital regions, such as the nerve synapse, where AChE is normally present. The test animals were AChE knockout mice.

Table 1.1. Protection against nerve agent toxicity by pretreatment with AChE or BChE.

Animal	Dose of BChE, mg	Dose of OP	Toxic signs	Reference
Monkey	39	2 LD_{50} soman	none	Broomfield (1991)
Monkey	39	1 LD_{50} sarin	none	Broomfield (1991)
Monkey	56	4 LD_{50} soman	none	Wolfe (1992)
Monkey	42.6	2 LD_{50} soman	behavioral decrement	Castro (1994)
Monkey	17 to 45	2 LD_{50} VX	none	Raveh (1997)
Monkey	17 to 45	3.3 LD_{50} soman	none	Raveh (1997)
Monkey	33 to 66	none	none	Matzke (1999)
Mouse	1.5	soman	survived	Ashani (1991)
Mouse	1.5	MEPQ	survived	Ashani (1991)
Mouse	4	1.5 LD_{50} VX	none	Raveh (1993)
Rat	4	1.5 LD_{50} soman	none	Raveh (1993)
Rat	4	1.5 LD_{50} soman	no cognitive deficits	Brandeis (1993)
Rat	10	MEPQ	response rate slowed	Genovese (1995)
Guinea pig	12	2 LD_{50} soman	none	Allon (1998)

Animal	Dose of AChE,mg	Dose of OP	Toxic signs	Reference
Monkey	7.5 and 15 mg	2.7 LD50 soman	none	Maxwell (1992)
Monkey	29 to 36 mg	5 LD50 soman	none	Wolfe (1992)
Mouse	0.9 mg	3 LD50 VX	survived	Wolfe (1987)
Mouse	0.5 mg	4 LD50 MEPQ	slight tremors	Raveh (1989)
Mouse	0.5 mg	2.6 LD50 soman	survived	Ashani (1991)
Mouse	0.5 mg	4.1 LD50 MEPQ	survived	Doctor (1991)
Mouse	2.4 mg	8 LD50 soman	slight cholinergic	Maxwell (1993)

Methods.

AChE knockout mice. A colony of AChE knockout mice is maintained at the University of Nebraska Medical Center. These animals were produced by gene targeting (Xie et al., 2000). All tissues have zero AChE activity and zero AChE protein. However, they have normal levels of BChE activity (Li et al., 2000).

Purified fetal bovine AChE was a gift from B.P. Doctor and Ashima Saxena at Walter Reed Army Institute of Medical Research. The enzyme had an activity of 12,000 units/ml. This was diluted with phosphate buffered saline to 130 units/ml and filter sterilized before injection into mice.

Treatment of mice with AChE. The dose of AChE to give to mice was estimated from the following facts. Wild-type mice have about 0.5 u/ml of AChE activity in serum. The total blood volume of a 6 gram mouse is 0.45 ml, that is,

7.5% of its body weight. 25% of the injected AChE activity was expected to remain in the blood 48 hours after injection (Raveh et al., 1989). Our goal was to give enough AChE to maintain a level of about 0.5 units/ml over a period of two days, so that mice would need to be injected only once every 2 days. Therefore, it was decided that the mice should receive 1 unit of AChE in a volume of 10 µl. This was expected to give the mice an initial AChE activity of 2 units/ml, which would drop to 0.5 units/ml after 48 hours.

Purified fetal bovine AChE was injected i.p. into 2 AChE -/- mice on postnatal days 11, 14, 16, 18, and 21. The injection volume was 10 µl and the activity of the AChE solution was 130 units/ml. On day 21, one mouse was perfused with saline through the heart, to flush blood from the brain. The brains were removed, homogenized in 0.5% Tween 20, 50 mM phosphate pH 7.4, centrifuged to remove tissue debris, and assayed for AChE activity (Li et al., 2000).

Mice were weighed daily and their body temperature was measured with a surface probe.

AChE activity assay. AChE activity was measured by the Ellman method (1961) in 1 mM acetylthiocholine. The buffer was 0.1 M potassium phosphate pH 7.0, at 25°C. A recording Gilford spectrophotometer interphased to a MacLab 200 (ADInstruments, Castle Hill, NSW, Australia) and a Macintosh computer was used. Samples were incubated with 0.1 mM iso-OMPA for 30 minutes to inhibit BChE, before being assayed for AChE activity.

Results

No bovine AChE activity in mouse brain. Bovine AChE activity was found at a low level (0.05 u/ml) in homogenate of mouse brain when the brain had not been perfused prior to being removed from the skull. However, perfused AChE -/- brain had no AChE activity. It was concluded that perfusion washed out the blood, and that the AChE found in unperfused brain was from the blood.

Bovine AChE activity in AChE -/- blood. By day 21, the mice had received 5 injections of bovine AChE (see Figure 1.1). On day 21, the activity of bovine AChE in the blood of AChE -/- mice was 3.8 units/ml. This shows that the AChE tetramers entered the blood from the intraperitoneal cavity, a result in agreement with Raveh et al., (1989).

Effect of AChE on phenotype. The AChE -/- mice had a noticeable body tremor, erratic movement, no righting reflex, low body temperature, and low body weight. Treatment with 5 doses of AChE over a period of ten days had no effect on this phenotype. Figure 1.1 compares the weight of AChE -/- mice treated with AChE, untreated AChE -/- mice, and normal littermates from postnatal days 11 to 21.

The surface body temperature of AChE -/- mice was about 33°C, whereas that of their littermates was about 35°C during postnatal days 13-18. Injection of AChE did not increase their body temperature.

No decrease in tremor, no change in movement, and no righting reflex was noticed in AChE -/- mice following treatment with AChE.

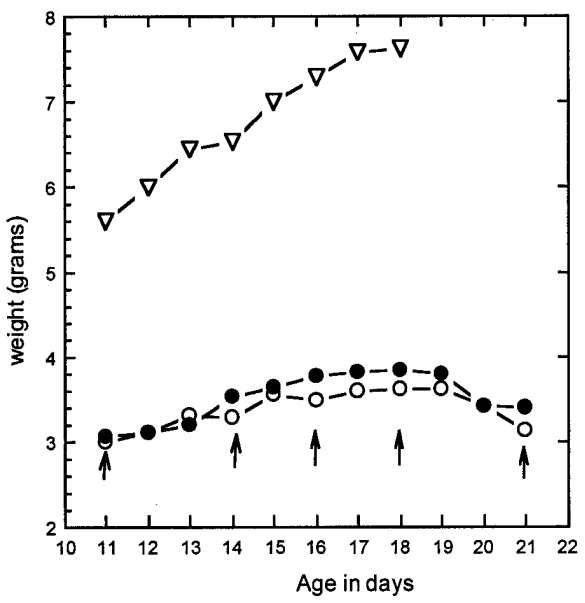

Figure 1.1. Injection of bovine AChE into AChE -/- mice. AChE -/- (n = 2) mice received 10 µl of 130 u/ml bovine AChE, i.p., on postnatal days 11, 14, 16, 18, and 21. AChE -/- mice weighed less than 4 g while their normal littermates (n = 11) weighed about twice as much. Injection of AChE did not improve weight gain.

Discussion

Fetal bovine AChE purified from plasma is a tetramer with a molecular weight of about 300,000 kDa (Ralston et al., 1985). AChE -/- mice treated with fetal bovine AChE had no AChE activity in their brain. Unperfused brain showed a low level of AChE activity, but this activity was in the blood. Perfused brain had zero AChE activity. These results show that a large glycoprotein of the size of AChE tetramers does not cross the blood brain barrier. This result confirms the well known fact that large charged molecules do not cross membranes and especially not the blood brain barrier. The significance of this observation is that treatment against nerve agent toxicity by injection of AChE or BChE enzymes, will be ineffective for doses of nerve agent that escape into the brain. Injected enzymes will scavenge nerve agents in the peripheral circulation but not in the brain.

The tremor in the AChE knockout mouse is caused by overstimulation of acetylcholine receptors by excess acetylcholine. The finding that tremor did not decrease following i.p. injection of AChE suggests that the acetylcholine was not accessible to the injected AChE. We conclude that the acetylcholine is

sequestered in the synapse at the nerve-muscle junction and cannot be acted on by exogenous AChE. Thus, the i.p. injected AChE does not cross the membranes into the nerve synapse.

The above provides two examples of the failure of AChE to cross membranes. Thus, it was surprising that AChE injected into the intraperitoneal cavity crossed membranes to enter the circulation. We confirmed the finding of others (Raveh at al., 1989; Ashani et al., 1991; Genovese and Doctor, 1995; Hoffman et al., 1996) that i.p. injected AChE and BChE were found in the blood. Similarly, Duysen et al. (2002) found that human BChE tetramers injected i.p. into mice entered the circulation within minutes.

Mouse serum contains 3 times more BChE than AChE activity (Li et al., 2000). An adult wild-type mouse has 1.9 u/ml BChE and 0.6 u/ml AChE activity (Li et al., 2000). BChE is highly capable of hydrolyzing acetylcholine. The tremor in AChE -/- mice is present despite the fact that AChE -/- mice have normal amounts of BChE. This means that the BChE is not in the right place to deplete the acetylcholine that causes the tremor. Like exogenously administered AChE, the endogenous BChE cannot get to the nerve synapse.

These results suggest an explanation for why exogenously administered native AChE and native BChE scavengers are limited in the level of protection they can provide. Nerve agent that escapes into the brain or other tissues will evade detoxication by AChE or BChE. A ceiling for protection has been reported by Doctor and others (see Table 1.1). No matter how much AChE or BChE was used for pretreatment, protection was afforded against a maximum of 8 LD50 doses of soman. The explanation for this ceiling of protection is that the nerve agent has entered compartments inaccessible to the exogenous scavenger enzymes.

Task 2. *A gene targeted mouse substituting the G177H mutant of human BChE for the ACHE gene will be made.*
2.1 A gene targeting construct will be made.

Task 2.1.

Abstract

The G117H mutant of human BChE has the unique capability of being able to hydrolyze the neurotransmitter acetylcholine and to hydrolyze organophosphorus (OP) toxicants. We want to know whether substitution of G117H BChE for AChE will make the mouse resistant to OP toxicity. Toward this end, we have constructed a gene-targeting plasmid to be used for making the G117H knockin mouse.

Introduction

Better protection from nerve agents would be achieved if an OP hydrolase were present in the nerve muscle junction and in neurons of the brain. Millard et al. (1995 and 1998) and Lockridge et al. (1997) have shown that the G117H mutant of human BChE hydrolyzed the OP agents sarin, VX, echothiophate, and paraoxon, as well as butyrylthiocholine and acetylthiocholine. Potentially, G117H BChE would perform the job that AChE normally performs, that of terminating nerve impulse transmission, with the advantage that G117H BChE is resistant to inactivation by OP. To test the idea that a mouse could be OP resistant, we are making a mouse that has the human G117H BCHE gene in the mouse ACHE gene locus.

Methods and Results

Design of gene targeting vector. We have designed and constructed the gene targeting vector to make the G117H knockin mouse. We designed the gene targeting vector so that expression of human G117H BChE would occur during development at the same time and in the same tissues as AChE in wild-type mice.

The design of the gene targeting vector aims to retain as much of the control regions of the ACHE gene as possible so as not to alter the expression pattern. Therefore, mouse ACHE intron 1, the mouse ACHE signal peptide, mouse ACHE intron 3, exon 4, intron 4, exon 5, intron 5, exon 6, and the 3' untranslated region are retained. The human BCHE in this vector is represented by amino acids Glu 1 to Gly 478 encoded by human BCHE exon 2. This plan is missing one potential control region, namely intron 2 of the ACHE gene. It was not possible to include intron 2 because it would have given unwanted recombination. Luo et al., (1998) have shown that retention of either intron 2 or 3 of mouse ACHE is sufficient to control exon 4 to exon 6 splicing. Since our construct contains intron 3, it is expected that normal control of splicing will be possible.

Structure of the gene targeting vector. See Figures 2.1 and 2.2. The gene targeting vector contains two selectable markers: the TK gene (2 kb) for negative selection, and the PGK-NEO gene (1.6 kb) for positive selection. The 34 nucleotide loxP sequence was placed on both sides of the NEO gene to allow deletion of the NEO gene by Cre recombinase.

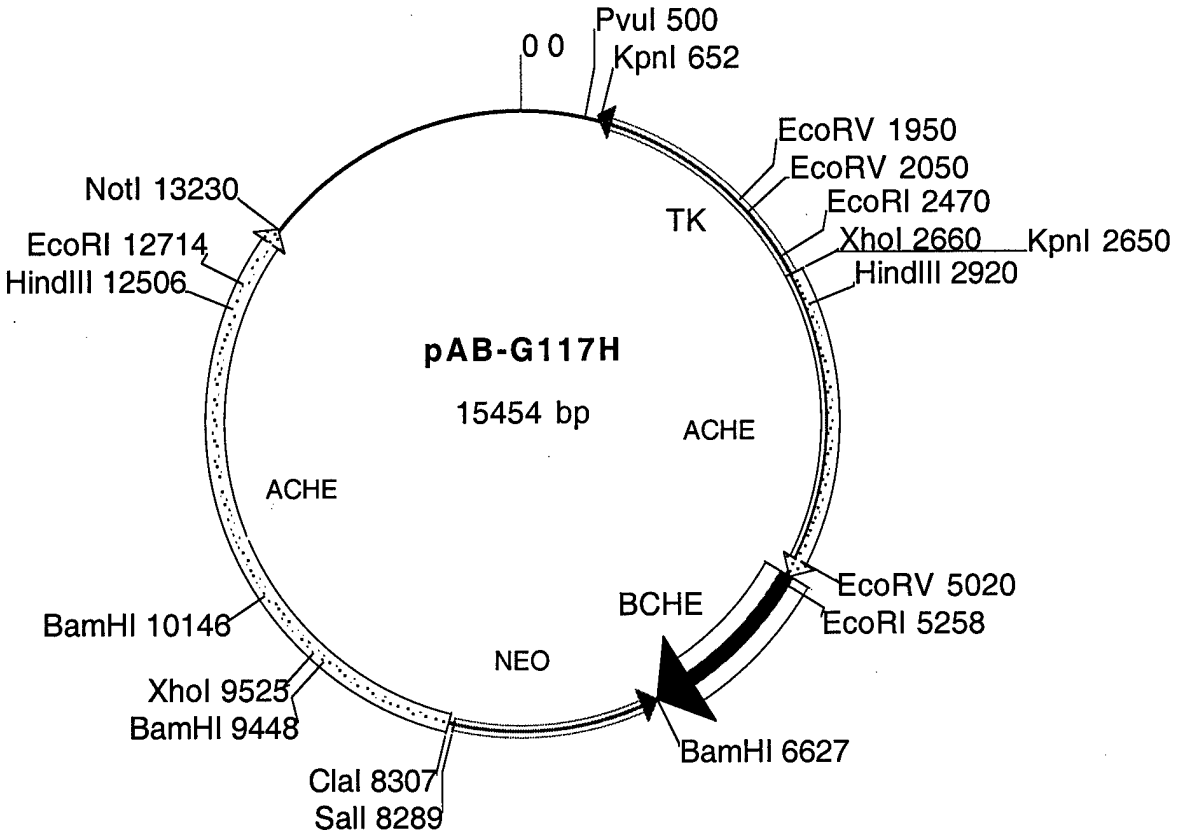

Figure 2.1. Map of the gene-targeting vector. The selectable markers are the TK gene (2 kb) and loxP NEO (1.66 kb). The 2502 bp of mouse ACHE are followed by 1433 bp of human BCHE, 1662 bp of loxP NEO, and 4923 bp of mouse ACHE. Human BCHE contains the G117H mutation.

The short arm of the gene targeting vector contains 2502 bp of mouse ACHE starting with 139 bp of intron 1b, followed by 292 bp of exon 1a, 226 bp of intron 1a, 84 bp of exon 1, 1644 bp of intron 1, and 115 bp of exon 2. The exon 2 portion of mouse ACHE includes the splice site and the codons for the 31 amino acid signal peptide of mouse ACHE. The mouse AChE signal peptide was

used rather than the human BChE signal peptide. The human BCHE gene starting with Glu+1 of the mature protein and ending at Gly 478 was placed immediately after the signal peptide. Gly 478 is at the end of exon 2 of human BCHE. A total of 1433 bp of the human BCHE gene are in this construct. At the end of exon 2 of human BCHE are 20 bp of intron 3 of mouse ACHE including the splice site. This is followed by the loxP PGK-NEO gene, and then by 4.9 kb of mouse ACHE. The 4.9 kb long arm contains 1340 bp of intron 3 of mouse ACHE, 170 bp of exon 4, 116 bp of intron 4, 128 bp of exon 5, 536 bp of intron 5, 122 bp of exon 6, and 3 kb of 3' untranslated region of mouse ACHE. The TK and NEO genes are oriented opposite to the direction of the ACHE-BCHE gene fusion. The sequence of the human BCHE gene was modified at residue Gly 117 to make the G117H mutant, because this mutant hydrolyzes organophosphorus toxicants including nerve agents.

The gene targeting vector was checked by DNA sequencing (Figure 2.2) and restriction enzyme digestion to be sure the construct was faithful to the design. The DNA sequence of the mouse ACHE portions was nearly identical to that in Genbank accession number NT_026533, even though our gene targeting vector was made from DNA of mouse strain 129SVj (Stratagene lambda FIX genomic library, catalog #946309) while the Genbank sequence is for mouse strain C57BL/6J. There were 5 differences: one nucleotide in intron 1a and 4 nucleotides in intron 1.

Cells that have undergone homologous recombination will be screened with a probe outside the targeted region. EcoRI digestion will give a 17 kb band for the wild-type and 6.7 kb band for the gene targeted allele. Bam HI digestion will give 6.5 and 6.2 kb bands.

Figure 2.2. Sequence of the gene targeting vector.

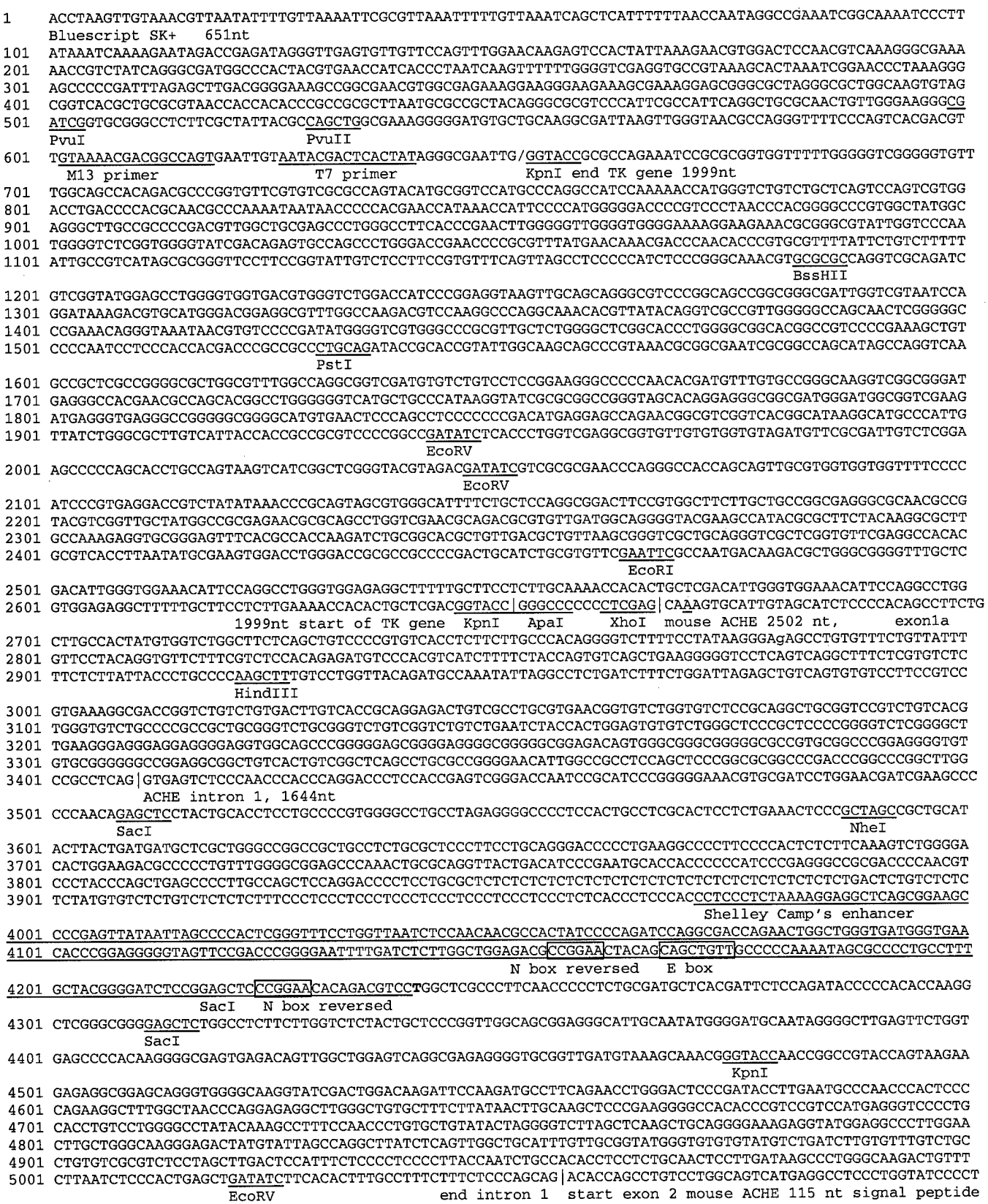

Figure 2.2 continued

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                                                                    end ACHE   +1aa human BCHE 1433 nt
5201 GGAAAAGTCAGAGGGATGAACTTGACAGTTTTTGGTGGCACGGTAACAGCCTTTCTTGGAATTCCCTATGCACAGCCACCTCTTGGTAGACTTCGATTCA
                                                                    EcoRI
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5501 TATGGATTTATGGTGGTCATTTTCAAACTGGAACATCATCTTTACATGTTTATGATGGCAAGTTTCTGGCTCGGGTTGAAAGAGTTATTGTAGTGTCAAT
                     G117H
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end exon2 BCHE intron3 20nt BamHI  loxP 34nt                                  end of NEO gene 1594nt
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  ClaI   ACHE 4923nt                KpnI  1340nt of ACHE intron 3
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9401 AGAAGAGAGTTGGGTGTTAAGAGTTGAAGAGGGTCCAGAGTCAGGAAAAGGATCCACGTCTTCATTCCTTATTCCTCTGGGCAGGCTGAGATTTCCCTTT
                                                    BamHI
9501 CCTTCCTCAG|GGACCCCAATGACCCTCGAGACTCCAAATCTCCACAGTGGCCACCGTACACCACTGCCGCGCAGCAATATGTGAGCCTGAACCTGAAGCC
              exon4 170nt    XhoI
9601 CTTAGAGGTGCGGCGGGGACTGCGCGCCCAGACCTGCGCCTTCTGGAATCGCTTTCTCCCCAAATTGCTCAGCGCCACCG|GTAGGCGCATGGAGTGGGGG
                                                                                    intron 4 116nt
9701 GAGCAGGGAATGCACAAGGCGGCAAGGGTTGGAAGGAGGGGAGAGAGATGGGGTGCAAAACACAGGGTGTAATCCCTCTCTTCTCCACCTCCCCAG|CCAC
9801 GGAGGCTCCTTGCACCTGCCCAAGCCCCGCCCATGGGGAGGCGGCCCCGAGGCCCGGGCCGGACTTAGCCCTGTCCCTCCTCTTCTTCCTTTTCCTCCTC
      exon 5 128nt
9901 CACTCCGGGCTTCCGTGGCTC|TAACTGCAATCCTTTCCCCTTCTGGTCTCAAAGCACACTTACTCTAGTGTGTAGCTGAGAAGGGGCCCCTCTCAGGGAT
                          intron 5 536nt
10001 ACAGAACCCCACTCCATTCTCAAGCCGAGACAAAATGGACAGACTCAGGGATCTTTGTTAAACAAAAATTCAAAAAACAGGATTTATTTTTTTTTTAATG
10101 TAAAAAATTATCCTTTGGAGCTTGAGCCTGGTGTTGAGGGGAGAAGGATCCGGGGCTAGTTGGCGTCAGCCAAAGGGCCTTATAATCGGGAACTATTTCT
                                                    BamHI
10201 GTATCTTCTCTCTTTTCCCCACTAACCCATGAACCCTTCTCCCACTCTGATCCTGTCCCTCCCCTCCGTCTTCCGGTGATTTTTATCTCTTCCTCTCCTT
10301 CCTGTTCCACCTCTCCCTTCATTCTCCGGATCATCCACTGTTCTCTTTCCACCACCCCGCTCGGCCTCCGACAATGCGTGTGTGCCGGCGCACTCACGAC
10401 CGGTCTCCACTACCCGACCTGTTCCGTTTCTCCCACTCTTGCGGGGTGCCCTGGCCGCAG|ATACTCTGGACGAGGCGGAGCGCCAGTGGAAGGCCGAGTT
                                                                  exon 6 mouse ACHE
10501 CCACCGCTGGAGCTCCTACATGGTGCACTGGAAGAACCAGTTCGACCACTATAGCAAGCAGGAGCGCTGCTCAGACCTGTGA|CCCCTTGGGGGACCCCAG
                SacI                                                                  end exon 6
```

Figure 2.2 continued

```
10601 GTCCTGCCGCCCTGCCCGAGCCCCTAGCTGTATATACACTATTTATTTAAGGGCTGGGATATAATACGACCGAGCCCCCAGGCCCTGTCCACTCCTCCCC
10701 GACTTCCTCCCACTAGGGGCTCCCCATCTTCTGCATGTCTTGGGCTAAGCTCCCCTCCCCGCGGtGCCTTCGCCCCTCTGGGCCGCCAATAAACTGTTAC
10801 AGCCACCAGAGTCTGTGCAACACGCGGGAAGTTGGGGCGGGGATGAGGTCAGAGACGCTGGGAACAGAAGCCTCGGGATTGGTGAGCACTGAGGCACCCT
10901 GCCCGCAGCATCCGGCGGAAGCAAGCGCTGGGCCCGCCCCTAGGGGCGGAGCCTGCGACTGCGCAGCAAGGCTGGTTTGCCGGCGCCTTTTAGAAGGCTC
11001 CTTAATTCGTCTGCCTGTGGAATCATCTCTCCTGCCGCTAGCCTGCTTGCTGAGAGTTCTCCGGTTCTACGTGCCCACACTGCCCGTAGCTAGTCCCAGA
                                        NheI
11101 AAACACCAGGAACCCGGAATTGGCCCTGCCTCGATGACAGCTGCTCTACCGAGCACCCTGGAGCTGCTGAAGGACGTGCACCTGGGCCTGCCTGTGCCCT
11201 GCCATGATCCCGCCCGACTGGCCCTCCTCTCAGGCCACTACCTTTACTATCACTATGGTTGCGATGGACTGGATGACCGTGGCTGGGGATGCGGCTACCG
11301 CACCCTGCAGACGCTGTGCTCCTGGCCAGGGGGCCAGTCCTCAGGCGTGCCTGGACTGCCAGCCTTGCAGGGAGCCCTAGAGGCCATGGGCGACAAGCCC
11401 CCCGGATTCCGGGGCTCCCGTAACTGGATCGGCTGTGTAGAGGCCAGTCTCTGCCTAGAACACTTCGGAGGACCTCAAGGGCGCCTATGCCACTTACCCC
11501 GCGGAGTAGGGCTTAGGGGAGAAGAGGAGCGGCTTTATTCACACTTTACAACGGGTGGGGGCCCAGTAATGGTAGGAGGAGATGCAGATGCCCAGTCCAA
11601 GGCCCTGCTGGGGATCTGTGAGGGGCCAGGTTCAGAAGTCTATGTCTTGATACTGGACCCACACTACTGGGGCACTCCAAAAAACCGTTGTGAACTACAA
11701 GCTGCTGGATGGGTGGGCTGGCAAAAGGTAAAAAGCGTCTTTGATTCCAATTCCTTCTACAACTTGTGCTTCACCAGAAATCTCTGAATCACCACGCCCT
11801 ACCTGCACCGTTGGGTAGATCCCAGAACTGAGACTAAACACAGCCACCTATTTACTTCGCACAGGCCCCCACCGCGATCAAGTTTGGGCCTGGCACAATG
11901 ATACGGGCAGGTGAATCTGAGTTCAAAGCCAACTTGGTCTACACAGCAAGGCCAAGCTGGAATTAGAGATACTGTTCTGAAAAATAATAAATTGGCGTAA
12001 TTCAGCTTACTATGGC|CCAGCCTGTGTTTGGGAAGGGACATACAGCACACAGTTAACACACACTACCAAAGTGTTTTTACTAGCGGGTAAAATAACGTAC
                      from Genbank NT_026533 mouse ACHE gene 1215 nt
12101 AAAAAACTTTTCAGCTGATGGGATAAAGTGGTCACGGGTACTGATGTAGCAATGCAGGAGGGGACATGGATGGCTCAAAAGAAGTCAACATCATCCGGGG
12201 CATCCAGGTCCCGATACTCCACTATGGCCCTTGGGTCTCCTCGAACCATCCTGTGAGATAAAGGAAATGTGAACAGATGTCCCTATAGTCAGAGACGGGG
12301 AAAGGCAGCCTGGGCATCGTTCGTGCTCACCTGTTCCGAGGTTTCCCAGGATAACCGCCTTGGCCTCGAAAAGCATCATAGTTCCCACGGCCGGCACCAT
12401 ATGGAGCATGGGGGTATGGAGGCCCTCCTGTTGGGACTGCAGGGCGGACAGCACCAGCTATGACAAAGACCCAGGGTGAACTAAAAACTGACCTCAACCT
12501 CAGCCTAAGCTTTGTAGCCGCCTACAGACACCAAACCCCTGCACTGCCTTTCCACACTCACCTCCATAGCCCAAGATGGGAGGCCGGGGCTGACCATACG
        HindIII
12601 GCATCAAGCCCTGTGGCGTCTGATGTGGGTAGGGAAGTCCTGGGGTCAGGCCTGGGGGGAGTACAACACGGACAGGAACATGAATTACTGCGGGGGAGGA
12701 AAGGGACTGACAAGAATTCTAGGAATAGGGCCTGAAGGTTAGTGGAGCACACTTCTCAAAGTAGAAGAAAGAAGTCAAAGGTATGGCAGCTACCACAGAT
                EcoRI
12801 CGTATCTTACTCTGGGCAGGGCCAGGTGGCTGAGCTGGCTTGATCTCAGGCAAAGCTGGGCGCTTGGCGTCTGTGAGAAAGTTATTGAAGAACGCCACCT
12901 CCTTCTTCACCTCCTCGATCTTCTCGGCATGCTTATTGAAGATATGCTTGCGCACAAACTCCGGGCCCTGGGAGGAGCGGATGAGGACGGACAGCATGCT
13001 GCAGTCTCTTCCCCACCGAACCCCGCTGGAAGGTAACAGCTTCCTCCAGGCTTTTAGGGGTTTTGCTACCCAAGCCCTCCTCCCAATCTACCCTCACAAT
13101 TCTTTTCACATCTCAGACCTTGAATTTCTTGCCACTGAGAGGACATAGCCACTTATCCTTGCCCAGTTCCTGCGTGTTGGAGGTGACAAACTTCTCCACT
13201 TCCTGCTCTGGGTCTTTTCGACCCATCTTC|GCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCGTAA
                    end ACHE gene  NotI BstXI SacII  SacI                T3 primer Bluescript SK+  2223nt
13301 TCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAAT
13401 GAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGG
13501 GAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAG
13601 GCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
13701 TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGC
13801 GTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCT
13901 CATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTAT
14001 CCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGG
14101 TGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGA
14201 GTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAG
14301 ATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGAT
14401 CCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACA|GTTACCAATGCTTAATCAGTGAGGCACCTATCTCA
                                                                    beta-lactamase gene 862nt
14501 GCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGA
14601 TACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTC
14701 CATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCA
14801 CGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCG
14901 GTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATG
15001 CTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCG
15101 CCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAAC
15201 CCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAG
15301 GGCGACACGGAAATGTTGAATACTCAT|ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGT
              end beta-lactamase
15401 ATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCC
```

Figure 2.2. DNA sequence of the gene targeting vector to make the G117H knockin mouse.

The parent vector is pBluescript SK+ (Stratagene). The total number of nucleotides in the gene targeting vector is 15,454 bp. The 2874 nucleotides of pBluescript, including the beta-lactamase gene for resistance to ampicillin, are between Not I (13230) and Kpn I (652). The 1999 nucleotides of the TK gene cassette are between KpnI 652 and KpnI 2650. The mouse ACHE gene begins

after XhoI 2660 and ends at 5170; these 2502 nucleotides of mouse ACHE include exon 1a, exon 1, intron 1 with Shelley Camp's enhancer sequence, and the start of exon 2. Only 115 nucleotides of mouse exon 2 are present and these encode the mouse ACHE signal peptide. The signal peptide is followed by 1433 nucleotides of the human BCHE gene. These 1433 bp encode the first amino acid of the mature BChE protein, Glu+1, to Gly 478 at the end of BCHE exon 2. Exon 2 of human BCHE includes G117H, and the catalytic triad residues Ser198, Glu325, and His438. 20 bp of intron 3 precede the NEO gene cassette. These intron 3 nucleotides are expected to allow splicing. The NEO gene cassette of 1594 bp is surrounded by loxP sites and inserted into BamHI 6627 and SalI 8289. Mouse ACHE gene sequences of 4923 bp are inserted between ClaI 8307 and NotI 13230. These 4923 bp include mouse ACHE intron 3, exon 4, intron 5, exon 5, intron 5, and exon 6.

Genbank accession number NT_026533 was used to verify the mouse ACHE gene sequences. Genbank J02224 has the HSV-TK sequence.

The N and E boxes in intron 1 are enhancers required for expression of AChE in muscle (Chan et al 1999; Luo et al., 1998; Angus et al., 2001).

Testing the human G117H BChE-mouse AChE chimeric enzyme. The cholinesterase that will be expressed in the G117H knockin mouse contains residues 1-478 of human BChE and residues 479-574 of mouse AChE. Thus, 83% of the amino acids are from human BChE and 17% are from mouse AChE. We designed a chimera because we wanted to retain as many of the control regions of the ACHE gene as possible, to maximize the possibility of mimicking the expression pattern of wild-type AChE in our G117H knockin mouse. Had we used BChE amino acids exclusively we would have had to delete intron 3, exon 5, and intron 5 of the ACHE gene.

Before making the mouse, we tested the chimeric protein for activity. This was done by making an expression plasmid that contained the signal peptide of mouse ACHE, followed by exon 2 of human BChE, exons 4 of mouse ACHE and exon 6 of mouse ACHE. See Figure 2.3.

```
1     AAGCTTAAGGTGCACGGCCCACGTGGCCACTAGTACTTCTCGAGCTCTGTACATGTCCGC
      Hind III
61    GGTCGCGACGTACGCGTATCGATGGCGCCAGCTGCAGGCGGCCGCCATATGCATCCTAGG
121   CCTATTAATATTCCGGAGTATACGTAGCCGGCTAACGTTAACAACCGGTACCTCTAGAAC
181   TATAGCTAGCATGCGCAAATTTAAAGCGCTGATATCTTCACACTTTGCCTTTCTTTCTCC
                                      EcoR V
241   CAGCAG|ACACCAGCCTGTCCTGGCAGTCATGAGGCCTCCCTGGTATCCCCTGCATACAC
end intron 1|start exon 2 mouse AChE signal peptide
301   CTTCCCTGGCTTTTCCACTCCTCTTCCTCCTCCTCTCCCTCCTGGGAGGAGGGGCAAGGG
361   CTGAAGATGACATCATAATTGCAACAAAGAATGGAAAAGTCAGAGGGATGAACTTGACAG
        Glu1 human BChE
421   TTTTTGGTGGCACGGTAACAGCCTTTCTTGGAATTCCCTATGCACAGCCACCTCTTGGTA
481   GACTTCGATTCAAAAAGCCACAGTCTCTGACCAAGTGGTCTGATATTTGGAATGCCACAA
541   AATATGCAAATTCTTGCTGTCAGAACATAGATCAAAGTTTTCCAGGCTTCCATGGATCAG
601   AGATGTGGAACCCAAACACTGACCTCAGTGAAGACTGTTTATATCTAAATGTATGGATTC
661   CAGCACCTAAACCAAAAAATGCCACTGTATTGATATGGATTTATGGTGGTCATTTTCAAA
                                                        G117H
721   CTGGAACATCATCTTTACATGTTTATGATGGCAAGTTTCTGGCTCGGGTTGAAAGAGTTA
781   TTGTAGTGTCAATGAACTATAGGGTGGGTGCCCTAGGATTCTTAGCTTTGCCAGGAAATC
841   CTGAGGCTCCAGGGAACATGGGTTTATTTGATCAACAGTTGGCTCTTCAGTGGGTTCAAA
901   AAAATATAGCAGCCTTTGGTGGAAATCCTAAAAGTGTAACTCTCTTTGGAGAAAGTGCAG
961   GAGCAGCTTCAGTTAGCCTGCATTTGCTTTCTCCTGGAAGCCATTCATTGTTCACCAGAG
1021  CCATTCTGCAAAGTGGTTCCTTTAATGCTCCTTGGGCGGTAACATCTCTTTATGAAGCTA
1081  GGAACAGAACGTTGAACTTAGCTAAATTGACTGGTTGCTCTAGAGAGAATGAGACTGAAA
1141  TAATCAAGTGTCTTAGAAATAAAGATCCCCAAGAAATTCTTCTGAATGAAGCATTTGTTG
1201  TCCCCTATGGGACTCCTTTGTCAGTAAACTTTGGTCCGACCGTGGATGGTGATTTTCTCA
1261  CTGACATGCCAGACATATTACTTGAACTTGGACAATTTAAAAAAACCCAGATTTTGGTGG
1321  GTGTTAATAAAGATGAAGGGACAGCTTTTTTAGTCTATGGTGCTCCTGGCTTCAGCAAAG
1381  ATAACAATAGTATCATAACTAGAAAAGAATTTCAGGAAGGTTTAAAAATATTTTTTCCAG
1441  GAGTGAGTGAGTTTGGAAAGGAATCCATCCTTTTTCATTACACAGACTGGGTAGATGATC
1501  AGAGACCTGAAAACTACCGTGAGGCCTTGGGTGATGTTGTTGGGGATTATAATTTCATAT
1561  GCCCTGCCTTGGAGTTCACCAAGAAGTTCTCAGAATGGGGAAATAATGCCTTTTTCTACT
1621  ATTTTGAACACCGATCCTCCAAACTTCCGTGGCCAGAATGGATGGGAGTGATGCATGGCT
1681  ATGAAATTGAATTTGTCTTTGGTTTACCTCTGGAAAGAAGAGATAATTACACAAAAGCCG
1741  AGGAAATTTTGAGTAGATCCATAGTGAAACGGTGGGCAAATTTTGCAAAATATGGG|GATC
                                    end exon 2 hBChE|start exon 4 mACHE
1801  CCAATGACCCTCGAGACTCCAAATCTCCACAGTGGCCACCGTACACCACTGCCGCGCAGC
1861  AATATGTGAGCCTGAACCTGAAGCCCTTAGAGGTGCGGCGGGGACTGCGCGCCCAGACCT
1921  GCGCCTTCTGGAATCGCTTTCTCCCCAAATTGCTCAGCGCCACCG|ATACTCTGGACGAGG
                                          end exon 4|start exon 6 mACHE
1981  CGGAGCGCCAGTGGAAGGCCGAGTTCCACCGCTGGAGCTCCTACATGGTGCACTGGAAGA
2041  ACCAGTTCGACCACTATAGCAAGCAGGAGCGCTGCTCAGACCTGTGACCCCTGGGGGCCC
                                          end exon 6          Apa I
```

Figure 2.3. Sequence of the chimeric G117H BChE-mouse AChE plasmid used for expression in cultured cells. The DNA for the mouse ACHE signal peptide (31 amino acids) is followed by exon 2 of human BCHE encoding 478 amino acids. Next are exons 4 and 6 of mouse ACHE. These 574 amino acids make the soluble tetrameric form of cholinesterase. The sequence is in the expression plasmid pGS.

The expression plasmid was transfected into 293T cells by calcium phosphate coprecipitation. The secreted enzyme was tested for activity with butyrylthiocholine and echothiophate. The activity with butyrylthiocholine and echothiophate was similar to the activity of the G117H BChE control. The control G117H BChE contains the human BChE sequence for the signal peptide and the 574 amino acids of the mature BChE protein (Millard et al. 1995; Lockridge et al., 1997). This positive result reassured us that the chimeric cholinesterase protein would be capable of hydrolyzing acetylcholine and OP in the G117H knockin mouse.

Discussion

We have completed the first step in the making of the G117H knockin mouse by constructing the gene targeting vector. This vector will be linearized by digestion with Not I and sent to a core facility for electroporation into R1 mouse embryonic stem cells. Positive colonies will be screened by us for homologous recombination by Southern blotting. The probe for Southern blots will be the same probe that was used by Xie et al. (2000) to screen for homologous recombination when we were making the ACHE knockout mouse.

Task 3. *A transgenic mouse that expresses human G117H BChE will be made.*
3.1 A plasmid will be made that contains the mouse ACHE promoter, mouse ACHE exon 1, and mouse intron 1 attached to the cDNA of human G117H BCHE.
3.2 The linearized, digested, and purified DNA will be microinjected into mouse fertilized eggs of strain FVB/N. The injected embryos will be transferred into pseudopregnant mice. The live pups will be tested for the presence of the transgene.
3.3 Mice that carry the transgene will be tested for expression of human G117H BCHE.
3.4 Founder mice expressing the highest levels of G117H BCHE will be mated to produce colonies of transgenic mice.
3.5 Transgenic mice will be characterized with respect to tissue location of the expressed transgene and the levels of expression.
3.6 Transgenic mice will be tested for resistance to OP.

Tasks 3.1, 3.2, 3.3, 3.4

Abstract

Transgenic mice are easier to make than gene targeted mice. The DNA construct is far less complicated, and no screening of embryonic stem cells is involved. Transgenic mice can be made in a year, whereas gene targeted mice take about 4 years to make. In this first year of the grant we made a transgenic G117H mouse. The 12 founder mice were bred to wild-type mice to test for germline transmission of the transgene. Eight founder mice produced transgenic offspring. The transgenic offspring are being bred to each other to produce a stable transgenic line carrying the maximum number of copies of the transgene. Blood from the founder mice has been tested for the presence of human BChE. No human BChE activity or human BChE protein has been detected so far. This analysis is still in progress.

Introduction

A transgenic mouse differs from a gene targeted mouse in that a transgenic mouse has its normal set of genes and in addition has one foreign gene. Multiple copies of the foreign gene, randomly integrate into a single or multiple chromosomal loci. There is no control of the site of integration. By contrast our gene targeted mouse has one copy of human BCHE in the ACHE gene locus.

The reason for making the G117H transgenic mouse is to find out whether this mouse is resistant to the toxic effects of OP. Can we protect the mouse from OP lethality and from cholinergic toxicity by introducing an OP hydrolyse gene?

The OP hydrolase is G117H BCHE because this mutant of human BChE hydrolyzes organophosphorus nerve agents and pesticides (Millard et al., 1995 and 1998; Lockridge et al., 1997).

Methods

Construction of the transgene. The ROSA26 promoter was selected because this promoter directs expression of the transgene in most tissue types throughout embryonic development and in adult tissues (Zambrowicz et al., 1997). A plasmid pBROAD (3.2 kb) containing the ROSA26 promoter was purchased from InvivoGen (San Diego, CA). A chimeric intron from pCI-neo (Promega) composed of the 5'-donor site from the first intron of the human beta-globin gene and the branch and 3'-acceptor site from the intron of an immunoglobulin gene heavy chain variable region (161 bp) was inserted into pBROAD between NcoI and Bgl II. The intron was added to pBROAD because the presence of an intron increases the number of animals that take up the transgene (Palmiter et al., 1991) and increases the level of expression of the transgene (Choi et al., 1991). The human BCHE cDNA encoded the 28 amino acid signal peptide and 574 amino acids of the full-length BChE protein. A single amino acid mutation at codon 117 substituted His for Gly to make the G117H mutation. Human BCHE was inserted into the Bgl II and Nhe I sites of pBROAD. A FLAG tag was placed at the 3' end of BCHE. The 3'untranslated region and polyadenylation sequence of the human elongation factor 1-alpha gene were in the pBROAD plasmid. The transgene was excised from the plasmid by digestion with Pac I to make a transgene of 3063 bp. See Figure 3.1.

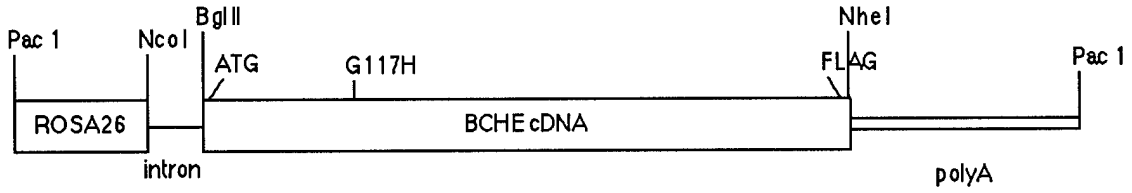

Figure 3.1. Schematic representation of the BCHE transgene. The ROSA26 promoter (382 bp) is followed by a chimeric intron (161 bp), the human BCHE cDNA (1859 bp) including a FLAG tag, and a poly A addition site (661 bp). The Pac I restriction site was used to produce a 3063 bp transgene for injection into fertilized mouse eggs.

The DNA sequence of the plasmid to make the transgenic G117H mouse is shown in Figure 3.2.

Figure 3.2. DNA sequence of G117H BCHE-FLAG in pBROAD

```
1     TAATGTGTTGGCGGACTGGCGGGACTAGGGCTGCGTGAGTCTCTGAGCGCAGGCGGGCGG
      Pac I    ROSA26 promoter U83173  366 bp
61    CGGCCGCCCCTCCCCCGGCGGCGGCAGCGGCGGCAGCGGCGGCAGCTCACTCAGCCCGCT
121   GCCCGAGCGGAAACGCCACTGACCGCACGGGGATTCCCAGTGCCGGCGCCAGGGGCACGC
181   GGGACACGCCCCCTCCCGCCGCGCCATTGGCCTCTCCGCCCACCGCCCCACACTTATTGG
241   CCGGTGCGCCGCCAATCAGCGGAGGCTGCCGGGGCCGCCTAAAGAAGAGGCTGTGCTTTG
301   GGGCTCCGGCTCCTCAGAGAGCCTCGGCTAGGTAGGGGATCGGGACTCTGGCGGGAGGGC
361   GGCTTGGTGACCGGTCACCATGGGCAG|GTAAGTATCAAGGTTACAAGACAGGTTTAAGGA
                        Nco I      start chimeric intron
421   GACCAATAGAAACTGGGCTTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCT
       sense primer
481   ATTGGTCTTACTGACATCCACTTTGCCTTTCTCTCCACAG|GTGTCCACTCCCAGATCTTC
                              end chimeric intron          Bgl II
541   GGAAGCCACCATGGATAGCAAAGTCACAATCATATGCATCAGATTTCTCTTTTGGTTTCT
            Met -28
601   TTTGCTCTGCATGCTTATTGGGAAGTCACATACTGAAGATGACATCATAATTGCAACAAA
                                            Glu1
661   GAATGGAAAAGTCAGAGGGATGAACTTGACAGTTTTTGGTGGCACGGTAACAGCCTTTCT
721   TGGAATTCCCTATGCACAGCCACCTCTTGGTAGACTTCGATTCAAAAAGCCACAGTCTCT
781   GACCAAGTGGTCTGATATTTGGAATGCCACAAAATATGCAAATTCTTGCTGTCAGAACAT
      W56W antisense primer
841   AGATCAAAGTTTTCCAGGCTTCCATGGATCAGAGATGTGGAACCCAAACACTGACCTCAG
901   TGAAGACTGTTTATATCTAAATGTATGGATTCCAGCACCTAAACCAAAAAATGCCACTGT
961   ATTGATATGGATTTATGGTGGTCATTTTCAAACTGGAACATCATCTTTACATGTTTATGA
                              G117H
1021  TGGCAAGTTTCTGGCTCGGGTTGAAAGAGTTATTGTAGTGTCAATGAACTATAGGGTGGG
1081  TGCCCTAGGATTCTTAGCTTTGCCAGGAAATCCTGAGGCTCCAGGGAACATGGGTTTATT
1141  TGATCAACAGTTGGCTCTTCAGTGGGTTCAAAAAAATATAGCAGCCTTTGGTGGAAATCC
1201  TAAAAGTGTAACTCTCTTTGGAGAAAGTGCAGGAGCAGCTTCAGTTAGCCTGCATTTGCT
1261  TTCTCCTGGAAGCCATTCATTGTTCACCAGAGCCATTCTGCAAAGTGGTTCCTTTAATGC
1321  TCCTTGGGCGGTAACATCTCTTTATGAAGCTAGGAACAGAACGTTGAACTTAGCTAAATT
1381  GACTGGTTGCTCTAGAGAGAATGAGACTGAAATAATCAAGTGTCTTAGAAATAAAGATCC
1441  CCAAGAAATTCTTCTGAATGAAGCATTTGTTGTCCCCTATGGGACTCCTTTGTCAGTAAA
1501  CTTTGGTCCGACCGTGGATGGTGATTTTCTCACTGACATGCCAGACATATTACTTGAACT
1561  TGGACAATTTAAAAAAACCCAGATTTTGGTGGGTGTTAATAAAGATGAAGGGACAGCTTT
1621  TTTAGTCTATGGTGCTCCTGGCTTCAGCAAAGATAACAATAGTATCATAACTAGAAAAGA
1681  ATTTCAGGAAGGTTTAAAAATATTTTTTCCAGGAGTGAGTGAGTTTGGAAAGGAATCCAT
1741  CCTTTTTCATTACACAGACTGGGTAGATGATCAGAGACCTGAAAACTACCGTGAGGCCTT
1801  GGGTGATGTTGTTGGGGATTATAATTTCATATGCCCTGCCTTGGAGTTCACCAAGAAGTT
1861  CTCAGAATGGGGAAATAATGCCTTTTTCTACTATTTTGAACACCGATCCTCCAAACTTCC
1921  GTGGCCAGAATGGATGGGAGTGATGCATGGCTATGAAATTGAATTTGTCTTTGGTTTACC
1981  TCTGGAAAGAAGAGATAATTACACAAAAGCCGAGGAAATTTTGAGTAGATCCATAGTGAA
2041  ACGGTGGGCAAATTTTGCAAAATATGGGAATCCAAATGAGACTCAGAACAATAGCACAAG
2101  CTGGCCTGTCTTCAAAAGCACTGAACAAAAATATCTAACCTTGAATACAGAGTCAACAAG
2161  AATAATGACGAAACTACGTGCTCAACAATGTCGATTCTGGACATCATTTTTTCCAAAAGT
2221  CTTGGAAATGACAGGAAATATTGATGAAGCAGAATGGGAGTGGAAAGCAGGATTCCATCG
2281  CTGGAACAATTACATGATGGACTGGAAAAATCAATTTAACGATTACACTAGCAAGAAAGA
2341  AAGTTGTGTGGGTCTCGACTACAAGGACGACGATGACAAGTAAGGGCCCGTTTAAACGCT
                        FLAG peptide DYKDDDDK
2401  AGCATTATCCCTAATACCTGCCACCCCACTCTTAATCAGTGGTGGAAGAACGGTCTCAGA
      Nhe I
2461  ACTGTTTGTTTCAATTGGCCATTTAAGTTTAGTAGTAAAAGACTGGTTAATGATAACAAT
2521  GCATCGTAAAACCTTCAGAAGGAAAGGAGAATGTTTTGTGGACCACTTTGGTTTTCTTTT
2581  TTGCGTGTGGCAGTTTTAAGTTATTAGTTTTTAAAATCAGTACTTTTTAATGGAAACAAC
2641  TTGACCAAAAATTTGTCACAGAATTTTGAGACCCATTAAAAAAGTTAAATGAGAAACCTG
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2701  TGTGTTCCTTTGGTCAACACCGAGACATTTAGGTGAAAGACATCTAATTCTGGTTTTACG
2761  AATCTGGAAACTTCTTGAAAATGTAATTCTTGAGTTAACACTTCTGGGTGGAGAATAGGG
2821  TTGTTTTCCCCCCACATAATTGGAAGGGGAAGGAATATCATTTAAAGCTATGGGAGGGTT
2881  TCTTTGATTACAACACTGGAGAGAAATGCAGCATGTTGCTGATTGCCTGTCACTAAAACA
2941  GGCCAAAAACTGAGTCCTTGGGTTGCATAGAAAGCTTCATGTTGCTAAACCAATGTTAAG
3001  TGAATCTTTGGAAACAAAATGTTTCCAAATTACTGGGATGTGCATGTTGAAACGTGGGTT
3061  AAT
      Pac I
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Figure 3.2. DNA sequence of G117H BCHE-FLAG in pBROAD between 2 Pac I sites (3063 nucleotides). The primers indicated above are used for screening mouse genomic DNA for the presence of the transgene. The primers are also used to make a probe for Southern blots. The probe is made by copying a single strand of the 392 bp PCR product with the aid of the antisense primer and P32-CTP.

Injection into fertilized mouse eggs and generation of transgenic mice. The DNA was microinjected into 164 fertilized (C57BL/6 X SJL)F2 eggs by the University of Michigan Transgenic Animal Model Core facility (Ann Arbor, MI) [Margaret Van Keuren mvkeuren@umich.edu , and Thomas Saunders tsaunder@umich.edu; http://www.med.umich.edu/tamc] and the eggs transferred into pseudopregnant recipient females. Out of 51 pups born, 13 carried the transgene. One founder mouse died before it could be bred, from bite injuries inflicted by other male mice.

Identification of mice that carry the transgene. DNA purified from tail snips with QIAamp DNA Mini Kit from Qiagen (Chatsworth, CA) was tested for the presence of the BCHE transgene by PCR. The sense primer matches the chimeric intron, the antisense primer matches human BCHE. The PCR product had 392 bp. The 34mer sense primer was 5'GGTTTAAGGAGACCAATAGAAACTGGGCTTGTCG, the 28mer antisense primer was 5'CCAAATATCAGACCACTTGGTCAGAGAC.

Southern blot. The purpose of Southern blotting was to identify transgenic mice with the highest number of copies of the transgene. Genomic DNA was purified and digested with EcoRI. Blots were probed with a single stranded P32-labeled probe containing 392 bases of the transgene. The location of the probe sequence is shown in Figure 3.2.

Assay for human BChE in mouse blood. Whole blood was collected from the leg vein of transgenic mice and centrifuged to separate the red cells from the serum. Three methods were used to test for the presence of human BChE. 1) The serum was assayed for butyrylcholinesterase activity with butyrylthiocholine by the Ellman method (Ellman et al., 1961). 2) A second method took advantage of the FLAG tag on the human BChE transgene. A 96 well plate coated with anti-FLAG antibody was purchased from Sigma (catalog # P2983).

3 to 30 µl of mouse serum from the test mice and from nontransgenic mice were placed into each well and allowed to bind to the anti-FLAG antibody. The positive control was culture medium containing a known amount of wild-type human BChE-FLAG. Wells were washed to remove unbound material, and then received butyrylthiocholine and DTNB to reveal BChE activity. 3) A third method was Western blotting. 5 to 11 µl of mouse serum were loaded per lane. Blots were hybridized with anti-FLAG conjugated to horse radish peroxidase (Sigma catalog # A8592). Use of this antibody avoided the use of secondary anti-mouse antibodies that would have crossreacted with endogenous mouse antibodies. Two positive controls were used: wild-type human BCHE-FLAG and carboxy-terminal FLAG-BAP Fusion Protein (Sigma catalog #P7457) 49.1 kDa.

Results

Transgenic founder mice. Out of 51 live births, 13 mice carried the human G117H BCHE gene. The mice were placed in quarantine at the University of Nebraska Medical Center for 6 weeks before being transferred to our care. During the quarantine period one mouse was mauled to death by other male mice in its cage.

The 12 founder mice have been bred to wild-type mice. To date 15 litters have been born. Of the 117 live pups (and 6 dead) in 15 litters, 24 pups were positive for the human BCHE transgene as determined by PCR.

Only 8 of the founder mice have produced positive pups. If all germ cells in a particular founder mouse contained the transgene, then 50% of pups should be transgenic. However, we have found only 20 to 30% of pups in a litter to be transgenic. This means the germ cells are a mixture of wild-type and transgenic.

Genomic DNA for Southern blots was prepared from the F1 generation, that is from pups born to founder mice.

Breeding of a transgenic female to a transgenic male of the same litter. Transgenic pups born to founder mice are by definition germline carriers of the transgene. To produce a transgenic line carrying the transgene on the maximum number of alleles, it is necessary to breed transgenic females to transgenic males of the same litter. Such a mating has produced 1 litter to date. The litter contained 6 pups. All pups died on postnatal day 3. Additional breeding is underway.

Transgene copy number. To identify the transgenic line with the highest copy number of the transgene, genomic DNA from transgenic pups in the F1 generation was analyzed on Southern blots. The pups selected for Southern blot analysis carried the transgene, as determined by the presence of the expected band of 392 bp in PCR assays. Pups in two litters gave a positive band on Southern blots (Figure 3.3B.). The highest band intensity was in pups from founder mouse F813T. It was estimated that F813T carried at least 10 copies of the transgene.

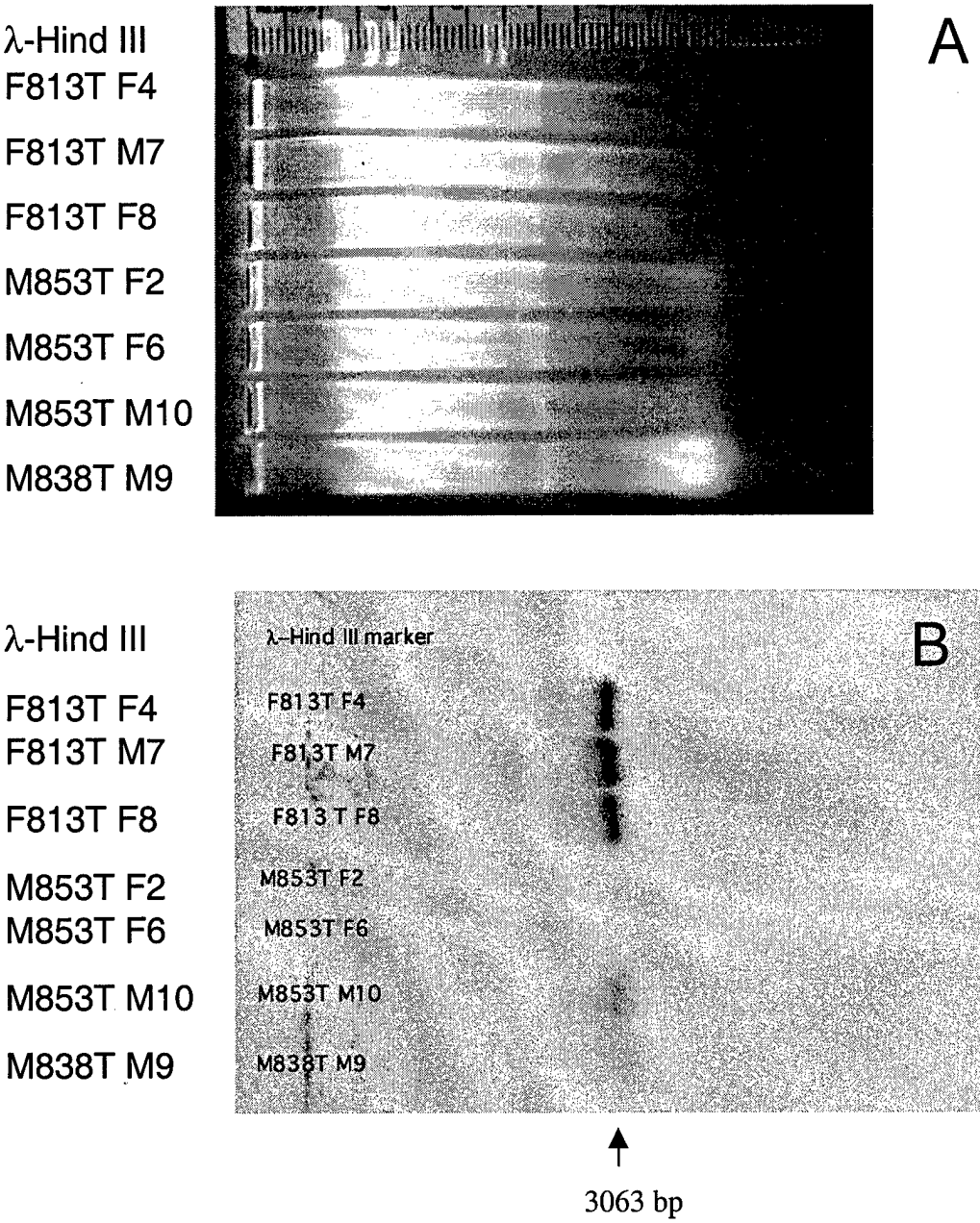

Figure 3.3. Southern blot of genomic DNA from transgenic mice. Panel A is an ethidium bromide stained agarose gel for genomic DNA digested with EcoRI. Panel B is the Southern blot hybridized with a probe specific for the transgene. The intense band has a size of 3063 bp. Two faint bands with a higher molecular weight are present in the F813T samples. These weaker bands represent a single copy of the transgene.

Human BChE activity and protein in mouse blood. A stable transgenic line has not yet been established from the founder mice. Such a line is being created but is not yet available. The mice that have been tested for human BChE do not carry the maximum number of transgenes.

Serum BChE activity in 12 founder mice and in 3 wild-type controls of the same strain and age is shown in Table 3.1.

Table 3.1. BChE activity in serum of transgenic founder mice.

Mouse	Sex	BChE activity, u/ml	Germline transmitter
F813 transgenic	F	2.04	yes
F825 transgenic	F	1.68	no
F829 transgenic	F	2.18	yes
F835 transgenic	F	1.93	yes
F855 transgenic	F	2.23	no
F857 transgenic	F	not tested	yes
F859 transgenic	F	2.45	yes
		Average 2.08	
M816 transgenic	M	1.39	no
M837 transgenic	M	1.18	no
M838 transgenic	M	1.25	yes
M844 transgenic	M	1.10	yes
M846 transgenic	M	1.16	dead 3/11/02
M853 transgenic	M	1.33	yes
		Average 1.23	
M842 wild-type	M	0.65	
M856 wild-type	M	1.31	
F860 wild-type	F	1.87	

The BChE activity in transgenic mouse blood may be slightly higher than in the control mice, but the number of control mice is too low to make a conclusion. More assays will be performed in the future.

Elisa assay with anti-FLAG antibody showed no human BChE in mouse serum. The positive control gave a strong yellow color, but the test samples had no color above background. The positive control contained 0.0076 units of activity per well. The lower limit of detection was 0.0015 units per well. The amount of mouse serum tested per well was 3 to 30 µl. The serum tested by Elisa was from founder mice. To get a yellow color in this test, the 3 µl of serum would have to contain 25 to 40% human BChE, while the 30 µl of serum would have to contain 2.5 to 4% human BChE. The absence of a positive result from the 30 µl sample suggests that this mouse had less than 0.05 u/ml of human BChE activity.

Western blots of mouse blood hybridized with anti-FLAG antibody showed a positive band in transgenic mice and in wild-type mice (Figure 3.4). The positive band had a molecular weight of about 90,000, which is the correct weight

for human BChE on an SDS gel. The presence of this band in wild-type control mice meant that the FLAG antibody was reacting nonspecifically with a protein in mouse serum. Therefore, Western blot results did not help answer the question of whether human BChE was expressed in the blood of our transgenic mice.

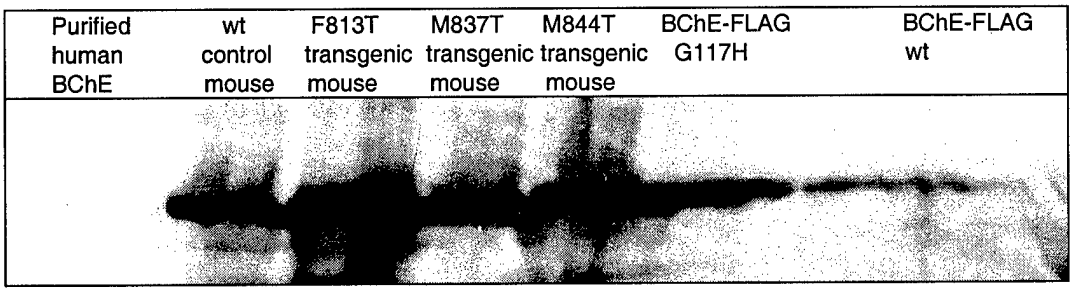

Figure 3.4. Western blot hybridized with anti-FLAG conjugated to horse radish peroxidase. The wild-type control mouse has a band at 90 kDa, at the same location as serum from the transgenic mice. The positive controls, G117H human BChE-FLAG and wild-type human BChE-FLAG, were in culture medium. The F813T founder mouse and her progeny have consistently given the strongest positive bands on Southern blots, leading to the expectation that this mouse has the best likelihood of expressing human BChE.

Discussion

The apparent absence of human BChE in the blood of our transgenic mice is not a final result. We are not yet ready to conclude that the transgene is not expressed. The mice tested to date have not had the maximum number of transgenes, because crossbreeding has not yet produced a stable transgenic line. In addition, we have not yet tested tissues other than blood for the presence of human BChE.

In the meantime we have started a second strategy for making a mouse that expresses human G117H BChE in all tissues during all times of development. This second strategy inserts the human G117H BCHE cDNA into the ROSA locus. Use of the ROSA locus is attractive because it has been successful for expressing other foreign proteins including beta-galactosidase, human placental alkaline phosphatase, green fluorescent protein, and site-specific recombinase FLPeR (Zambrowicz et al., 1997; Kisseberth et al., 1999; Soriano, 1999; Farley et al., 2000; Awatramani et al., 2001). Insertion of human BCHE into the ROSA locus requires construction of a gene targeting vector.

Task 4. *Gene therapy with AChE.*
4.1 Human AChE cDNA will be cloned into a shuttle vector. The linearized shuttle vector and pAdEasy-1 will be cotransfected into bacteria to allow homologous recombination. Colonies resistant to kanamycin will be screened by restriction endonuclease digestion.
4.2 The adenoviral vector containing human AChE, pAd-ACHE, will be linearized and transfected into 293 cells. Virus production will be visualized by fluorescence of green fluorescence protein and by measuring AChE activity.
4.3 Viral stocks will be amplified in 293 cells to obtain 10^{11} to 10^{12} plaque forming units. The virus will be purified in preparation for injection into mice.
4.4 Mice will be injected intravenously with various doses of adenoviral vector. The site of localization of the adenovirus will be determined. Expression levels of AChE will be determined. The duration of expression of AChE will be measured.

Tasks 4.1, 4.2, and 4.3

Abstract

Adeno-associated virus (AAV) is attractive as a gene transfer vector because it has no known pathogenicity, it is minimally immunogenic, and it can transduce nondividing cells. Use of AAV is approved by the Food and Drug Administration for treatment of a variety of diseases. For these reasons we selected AAV for gene therapy trials. The AChE knockout mouse will be treated with a virus that delivers AChE. To date our progress includes construction of the viral expression plasmid (task 4.1), production of the virus (task 4.2), and purification of the virus (task 4.3).

Introduction

The AChE -/- mouse can be used as a model for testing the effectiveness of gene therapy to deliver the ACHE gene. The delivery of AChE via gene therapy is expected to have military applications for treatment and protection against OP.

Methods

Preparation of adeno-associated virus construct carrying the human ACHE gene. The human ACHE gene was cloned into the pSCMF4 plasmid (BCCM™/LMBP Universiteit Gent, Belgium) for the purpose of acquiring an EcoRI site at the 5' end. The 1.9 kb piece was digested with EcoRI and SalI and cloned into the pAAV-MCS plasmid (Stratagene, catalog #240071) in ultracompetent XL10-Gold cells (Stratagene catalog #200314). The recombinant expression plasmid was co-transfected into HEK293 cells with pHelper (carrying adenovirus derived genes) and pAAV-RC (carrying AAV-2 replication and capsid genes). These three plasmids together supply all the trans-acting factors required for AAV replication and packaging in HEK293 cells. A kit from

Stratagene includes the plasmids AAV-MCS, pHelper, and pAAV-RC (Stratagene, catalog #200314). The Stratagene kit is based on work from the laboratory of Richard Samulski (Xiao et al., 1998).

Purification of adeno-associated virus. Culture medium and cells were harvested 72 hours post-transfection in HEK293 cells (Amiss and Samulski, 2001). Virus was released from the cells by freeze-thawing three times. 500 ml of viral suspension was partially purified and concentrated by ammonium sulfate precipitation. 25 ml of viral suspension was overlayed on a cesium chloride gradient (Haberman et al., 1999) and centrifuged for 48 h at 288,000 x g (41,000 rpm in a Beckman SW-41 Ti rotor). The tube was punctured with a needle and 0.5 ml fractions collected. Fractions were tested for the presence of viral DNA on a dot blot hybridized with a P32 labeled probe for human ACHE. Fractions containing the highest concentration of virus were pooled and frozen at –80°C.

A procedure for estimating the viral titer was developed. HEK293 cells were infected with the virus. 24 hours later, cells were fixed with glutaraldehyde and formaldehyde (Amiss and Samulski, 2001) and stained for AChE activity by the method of Karnovsky and Roots (1964). Uninfected cells served as the negative control for background staining.

A second plasmid expressing human ACHE tagged with the FLAG epitope at the C-terminal was constructed. The purpose of this second expression plasmid was to aid in detection of the recombinant human AChE protein in mouse tissues. The FLAG antibody is available conjugated to horse radish peroxidase. This makes it unnecessary to use an anti-mouse IgG secondary antibody and therefore reduces the background staining.

Results

Results to date include production and purification of the virus. We have not yet injected the virus into animals.

Discussion

We have completed tasks 4.1, 4.2 and 4.3. No animal experiments have been done with the virus as of this date. We will provide a discussion of results after we know what happens to the AChE knockout mouse as a result of gene therapy with the AAV-ACHE virus.

Key Research Accomplishments

• The creation of two test animals is in progress. These animals will be used to test the protection afforded by foreign genes against nerve agent toxicity. 1) The gene targeting vector for making the G117H knockin mouse has been made. 2) A transgenic mouse containing multiple copies of the human butyrylcholinesterase cDNA has been made.
• A gene therapy protocol using adeno-associated virus to deliver acetylcholinesterase is being developed.

Reportable Outcomes

Published manuscript

Duysen EG, Bartels CF, Lockridge O. Wild-type and A328W mutant human butyrylcholinesterase tetramers expressed in Chinese Hamster Ovary cells have a 16-hour half-life in the circulation and protect mice from cocaine toxicity. J Pharmacol Exp Ther 302: 751-758 (2002)

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AChE knockout mice

A colony of AChE knockout mice that lives to adulthood has been established.

Conclusions. Summary of results to include the implications of the research.

The methods developed in this first year of the grant provide the groundwork for achieving the goals of this research. Our goal is to provide new therapeutics, in the form of genes and proteins, for protection against organophosphorus nerve agents.

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Appendices

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Wild-Type and A328W Mutant Human Butyrylcholinesterase Tetramers Expressed in Chinese Hamster Ovary Cells Have a 16-Hour Half-Life in the Circulation and Protect Mice from Cocaine Toxicity

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ABSTRACT

Human butyrylcholinesterase (BChE) hydrolyzes cocaine to inactive metabolites. A mutant of human BChE, A328W, hydrolyzed cocaine 15-fold faster compared with wild-type BChE. Although the catalytic properties of human BChE secreted by Chinese hamster ovary (CHO) cells are identical to those of native BChE, a major difference became evident when the recombinant BChE was injected into rats and mice. Recombinant BChE disappeared from the circulation within minutes, whereas native BChE stayed in the blood for a week. Nondenaturing gel electrophoresis showed that the recombinant BChE consisted mainly of monomers and dimers. In contrast, native BChE is a tetramer. The problem of the short residence time was solved by finding a method to assemble the recombinant BChE into tetramers. Coexpression in CHO cells of BChE and 45 residues from the N terminus of the COLQ protein yielded 70% tetrameric BChE. The resulting purified recombinant BChE tetramers had a half-life of 16 h in the circulation of rats and mice. The 16-h half-life was achieved without modifying the carbohydrate content of recombinant BChE. The protective effect of recombinant wild-type and A328W mutant BChE against cocaine toxicity was tested by measuring locomotor activity in mice. Pretreatment with wild-type BChE or A328W tetramers at a dose of 2.8 units/g i.p. reduced cocaine-induced locomotor activity by 50 and 80%. These results indicate that recombinant human BChE could be useful for treating cocaine toxicity in humans.

Human BChE has a major role in detoxication of cocaine (Kalow and Grant, 2001). Treatment of rodents and cats with human or horse BChE protects from cocaine-induced hypertension, cardiac arrhythmia, hyperactivity, seizures, and lethality (Hoffman et al., 1996; Lynch et al., 1997; Mattes et al., 1997; Carmona et al., 1998). The amount of BChE present in human blood and tissues is insufficient to instantly detoxify inhaled or injected cocaine because BChE hydrolyzes the (−)-cocaine isomer slowly. By contrast, the (+)-cocaine isomer is hydrolyzed 2000- fold faster (Gatley, 1991; Xie et al., 1999) and has none of the physiological effects of (−)-cocaine. The lack of pharmacologic activity of (+)-cocaine is attributed to rapid hydrolysis by BChE (Gatley, 1991). A BChE with a faster rate of hydrolysis might render (−)-cocaine pharmacologically inactive.

One purpose of this work was to increase the catalytic efficiency of human BChE for hydrolysis of (−)-cocaine. A 4-fold increase in catalytic efficiency had been achieved with the A328Y mutant (Xie et al., 1999). In this report a further increase in catalytic efficiency is provided by the A328W mutant, which hydrolyzes (−)-cocaine 15-fold faster than does wild-type BChE. The laboratory of Stephen Brimijoin has engineered other cocaine-hydrolyzing mutants of BChE (Sun et al., 2001). A large aromatic residue at position 328 is expected to orient (−)-cocaine into position for attack by the active site serine (Xie et al., 1999).

A second goal was to produce the A328W mutant BChE in a form that would have a long residence time in the circulation of animals. Saxena et al. (1998) had shown that recombinant human BChE had a very short residence time in the circulation of mice, on the order of minutes, whereas native human BChE purified from plasma had a mean residence time of 46 h. A significant difference between recombinant and native BChE was that recombinant human BChE se-

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ABBREVIATIONS: BChE, butyrylcholinesterase; CHO, Chinese hamster ovary; AChE, acetylcholinesterase; RSV, Rous sarcoma virus; G418, geneticin; 293 cell, human embryonic kidney 293cell.

creted by Chinese hamster ovary (CHO) cells consisted mainly of monomers and dimers (Blong et al., 1997; Saxena et al., 1998), whereas native BChE is a tetramer. The finding by Bon et al. (1997) and Krejci et al. (1997) that a proline-rich peptide from the N terminus of the collagen-tail protein (COLQ gene) caused assembly of acetylcholinesterase into tetramers led us to the experiments that solved the problem. Coexpression of BChE with a 45-amino acid peptide encoded by the COLQ gene converted 70% of the recombinant BChE to tetramers (Altamirano and Lockridge, 1999). Tetrameric recombinant BChE was found to have a residence half-time of 16 h in the circulation of rats and mice. This long half-time was achieved without modifying the carbohydrate content.

A third goal was to demonstrate the protective effect of recombinant human BChE against cocaine toxicity. Native wild-type BChE, purified from human or horse plasma, has previously been shown to protect mice, rats, and cats from cocaine toxicity (Hoffman et al., 1996; Lynch et al., 1997; Mattes et al., 1997; Carmona et al., 1998). However, recombinant human BChE has not previously been tested in animals. The protective effect of recombinant BChE tetramers was shown by measurement of locomotor activity in mice. Cocaine at a dose of 25 mg/kg i.p. induced high locomotor activity, but pretreatment with BChE reduced activity by 50 to 80%. The A328W mutant was more effective than wild-type BChE at reducing locomotor activity. These results indicate that recombinant wild-type and mutant BChE may be useful for treatment of cocaine toxicity in humans.

Materials and Methods

Mutagenesis. The mutation A328W was introduced into human BChE by polymerase chain reaction as previously described (Xie et al., 1999). The cloned cDNA was completely sequenced to verify the presence of the A328W mutation and the absence of unwanted mutations.

Expression of Human BChE. Human BChE was expressed in Chinese hamster ovary cells (CHO-K1; ATCC 61-CCL), stably transfected with plasmid pGS-BCHE wild-type or A328W as previously described (Xie et al., 1999). Selective pressure to retain the plasmid was provided by 50 μM methionine sulfoximine in the initial period and reduced to 25 μM for maintenance. Secreted BChE was collected into serum-free and glutamine-free culture medium, Ultraculture (BioWhittaker, Walkersville, MD, catalog number 12-725B), thus avoiding contamination by AChE present in fetal bovine serum. No antibiotics were added to the culture medium. The cells were grown in 1-liter roller bottles. Culture medium (150 ml per bottle) in the roller bottles was changed every 2 to 4 days. A roller bottle yielded BChE continuously for as long as 6 months. Each liter of culture medium contained 1 to 5 mg of BChE.

N Terminus of the Collagen-Tail. The proline-rich attachment domain is a 17-residue peptide from the N terminus of the collagen-tail encoded by the COLQ gene (Bon et al., 1997; Krejci et al., 1997). It has two cysteines as well as five and three consecutive prolines in the sequence CCLLMPPPPPLFPPPFF. Bon et al. (1997) first reported that this peptide caused recombinant AChE monomers to assemble into tetramers. A clone encoding 117 amino acids of the N terminus of the rat collagen-tail was a gift of Dr. Eric Krejci. We modified this clone by PCR. The modified collagen-tail was called rQ45; it included 22 codons for the signal peptide, 45 for the N terminus of COLQ, and 8 for the FLAG epitope DYKDDDDK cloned into the mammalian expression plasmid, pRc/RSV (Invitrogen, Carlsbad, CA) (see Fig. 1). The expression plasmid for rQ45 had a different promoter from the promoter for expression of BChE so that the two would not compete for transcription factors. The promoter for expression of rQ45 was Rous sarcoma virus long terminal repeat, whereas the promoter for expression of BChE was cytomegalovirus. The selectable marker in plasmid pRc/RSV-rQ45 is the NEO gene and in plasmid pGS is glutamine synthetase.

```
                    signal peptide
HindIII      M  A  V  L  N  P  M  T  L  G  I  Y
AAGCTTGCCACCATGGCTGTCCTGAATCCAATGACTTTGGGAATTTAT
                                 1
 L  Q  L  F  F  C  S  I  V  S  Q  P  T  F  I  N
CTCCAACTCTTCTTCTGCTCCATCGTGTCGCAGCCAACTTTCATCAAC

 S  V  L  P  I  S  A  A  L  P  G  L  D  Q  K  K
AGTGTCCTCCCAATCTCAGCAGCCCTTCCTGGCCTGGATCAGAAGAAA
                    proline rich attachment domain
 R  G  N  H  K  A  C  C  L  L  M  P  P  P  P  P
CGAGGCAACCACAAAGCATGCTGCCTACTGATGCCCCCGCCACCCCCA
                    45  FLAG epitope
 L  F  P  P  P  F  F  D  Y  K  D  D  D  D  K  *
CTCTTCCCACCGCCATTCTTCGACTACAAGGACGACGATGACAAGTGA
 *  *  NotI
TAATAGCGGCCGC
```

Fig. 1. Sequence of rQ45. The modified collagen-tail peptide was called rQ45; it included 22 codons for the signal peptide, 45 for the rat COLQ gene (Krejci et al., 1997), and 8 for the FLAG epitope DYKDDDDK cloned into the mammalian expression plasmid, pRc/RSV (Invitrogen) at the *Hin*dIII and *Not*I sites. Coexpression of rQ45 with BChE yields BChE tetramers.

Coexpression of Human BChE and rQ45. The tetramerization domain of human BChE is located at the C terminus where the 40 amino acids encoded by exon 4 are essential for formation of dimers and tetramers (Blong et al., 1997; Altamirano and Lockridge, 1999). Full-length BCHE cDNA encoding 574 amino acids of the mature protein was used for expression. Stable CHO cell lines expressing wild-type or A328W human BChE were transfected with pRc/RSV-rQ45. Clones expressing both BChE and rQ45 were selected in Ultraculture containing 25 μM methionine sulfoximine and 0.8 mg/ml G418 (geneticin). Cells were amplified in T150 flasks and finally in roller bottles for large-scale production of tetrameric BChE. After cells had coated the roller bottle, the G418 was no longer added to culture medium.

Purification of Recombinant BChE. Serum-free culture medium was collected from roller bottles over a period of months. The BChE-containing culture medium was stored at 4°C in sterile bottles. Ten to 20 liters of culture medium containing 50 to 100 mg of BChE were filtered through Whatman filter paper no. 1 (Whatman, Clifton, NJ) on a Buchner funnel, or through a coffee filter on a fritted glass funnel attached to a water aspirator, to remove cell debris. The filtered culture medium was loaded onto a 300- to 400-ml procainamide-Sepharose affinity column packed in a Pharmacia column XK50/30 (Pharmacia, Peapack, NJ). This column has a diameter of 5 cm, allowing a flow rate of 1 liter/h. All of the BChE activity was retained by the affinity gel. The column was washed with 20 mM potassium phosphate, 1 mM EDTA, pH 7, until the absorbance at 280 nm of the eluate was nearly zero. When the BChE on the column was wild-type BChE, the column was washed with 0.2 M NaCl in buffer to elute contaminating proteins. When the BChE was A328W, the column was washed with 0.6 M NaCl in buffer because A328W remained bound to the affinity gel at this salt concentration. The column was washed with buffer before eluting wild-type BChE with 1 M NaCl or A328W with 2 M NaCl containing 0.2 M choline chloride in 20 mM potassium phosphate, pH 7. The yield of BChE from this first step was 90 to 100%.

BChE can be eluted from the affinity column with inhibitors or poor substrates rather than NaCl. For example the following have been found to work: 0.2 M procainamide, 0.2 M procaine, 0.2 M decamethonium, 0.2 M acetyl-β-methylcholine, 0.2 M tetramethylammonium bromide, and 0.2 M succinyldicholine. A good substrate such as 0.2 M acetylcholine also elutes the enzyme, but the pH rapidly drops below 4 due to the release of acetic acid by hydrolysis

of acetylcholine, and the low pH can inactivate the BChE if exposure is prolonged. We generally choose to elute with NaCl because inhibitors cannot be removed completely from BChE. This is a concern if the BChE is to be used for injection into humans.

The BChE was dialyzed against 20 mM Tris-Cl, pH 7.4, to reduce the salt concentration and then loaded onto 400 to 500 ml of DE52 (Whatman) ion exchanger packed in a Pharmacia XK50/30 column. The column was washed with 20 mM Tris-Cl, pH 7.4, until the absorbance of the eluate was nearly zero. BChE was eluted with 0.15 M NaCl in 20 mM Tris-Cl, pH 7.4. The BChE eluted as a shoulder ahead of a contaminating peak. The yield of BChE from this second chromatography step was about 70%. The cleanest fractions were 80 to 90% pure. Purity was estimated from specific activity and from gel electrophoresis. A specific activity of 720 units/mg was the standard for 100% pure wild-type BChE. Units of activity were measured with 1 mM butyrylthiocholine in 0.1 M potassium phosphate, pH 7.0, at 25°C. Protein concentration was measured by absorbance at 280 nm, where an absorbance of pure BChE, at 1 mg/ml, was 1.8.

Purified recombinant human BChE was dialyzed against phosphate-buffered saline and concentrated to 1 mg/ml in a Millipore Diaflo apparatus (Millipore Corp., Bedford, MA) fitted with a PM10 (particles < 10 μm in diameter) membrane. The dialyzed, concentrated BChE was filter-sterilized through a 0.2-μm filter and stored at 4°C. Although dilute BChE loses activity when it is frozen in the absence of a cryoprotectant such as glycerol, BChE concentrated to 1 mg/ml in phosphate-buffered saline can be frozen without loss of activity.

Purification of BChE from Human Plasma. Native BChE was purified from human plasma by ion-exchange chromatography at pH 4.0, followed by affinity chromatography on procainamide Sepharose.

Affinity Gel. Procainamide-Sepharose 4B affinity gel, with a 6 carbon spacer, was custom made by Yacov Ashani at the Israel Institute for Biological Research, Ness-Ziona, Israel. The concentration of bound procainamide was estimated to be 34 μmol/ml. Used affinity gel was recycled by washing on a fritted glass funnel with 0.5 M glacial acetic acid, followed by water. The washed gel was stored in the presence of 20% ethanol at 4°C. The gel has been reused repeatedly for several years, with no apparent loss in binding capacity.

k_{cat} and K_m for Cocaine, Butyrylthiocholine, and Benzoylcholine. (−)-Cocaine hydrochloride (purchased from Sigma-Aldrich, St. Louis, MO, after obtaining a controlled substance license from the U.S. Department of Justice) was dissolved in water to make a 0.1 M stock containing 34 mg/ml. Aliquots were frozen at −80°C, thawed once, and discarded. The rate of hydrolysis of (−)-cocaine was measured in the spectrophotometer at 240 nm, using an extinction coefficient of 6700 M^{-1} cm^{-1} for the difference in absorbance between cocaine and benzoic acid (Gatley, 1991). The temperature was 25°C, and the buffer was 0.1 M potassium phosphate, pH 7.0.

Butyrylthiocholine and benzoylcholine k_{cat} and K_m were measured in 0.1 M potassium phosphate buffer, pH 7.0, at 25°C as described (Xie et al., 1999). The K_m was determined for benzoylcholine concentrations ranging from 12.5 to 60 μM.

Purified recombinant BChE was titrated with chlorpyrifos oxon (Chem Service Inc., West Chester, PA) for determination of active site concentration. Maximum rate of hydrolysis per active site, denoted as k_{cat}, was calculated by dividing V_{max} by the concentration of active sites.

BChE Activity Assay. Serum samples were tested for BChE activity with 1 mM butyrylthiocholine and 0.5 mM 5,5′-dithio-bis-(2-nitrobenzoic acid), in 0.1 M potassium phosphate buffer, pH 7.0, at 25°C. A temperature-controlled Gilford spectrophotometer that interfaced via a MacLab data recorder (ADInstruments Pty Ltd., Castle Hill, Australia) to a Macintosh computer (Apple, Cupertino, CA) was used. Formation of the product was followed by the absorbance increase of 5-thio-2-nitrobenzoic acid at 412 nm, using a molar extinction coefficient of 13,600 M^{-1} cm^{-1} (Ellman et al., 1961). Activity is reported as units per milliliter, where 1 unit represents the hydrolysis of 1 μmol of butyrylthiocholine per min.

Nondenaturing Gel Electrophoresis. The relative amount of tetramers, dimers, and monomers was estimated on activity-stained nondenaturing polyacrylamide gels. Four to 30% polyacrylamide gradient gels were prepared in a Hoeffer SE600 gel apparatus (Hoeffer, San Francisco, CA; presently part of Pharmacia, Inc.). Electrophoresis was at 120 V constant voltage for 15 h at 4°C. It was important to keep the gels cold during electrophoresis to avoid degrading the heat-labile monomers and dimers (Blong et al., 1997). Gels were stained for BChE activity in the presence of 2 mM butyrylthiocholine iodide by the method of Karnovsky and Roots (1964). Band intensity was quantified with a BioImage 110S System (Millipore).

Elimination Time of Human BChE from Rats and Mice. Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health. Five male Sprague-Dawley rats weighing approximately 350 g were anesthetized by intraperitoneal administration of 60 mg/kg α-chloralose and 800 mg/kg urethane. A cannula made of Silastic tubing was implanted into the jugular vein. Blood for the zero time point was collected. Human BChE was delivered through the cannula to a dose of 1 mg/kg; that is, 0.35 ml of 1 mg/ml BChE (720 U/ml for wild-type BChE) was infused over a 45-s period. The cannula was flushed with 0.1 ml of saline, and a blood sample was collected 1 min after the flush with saline. Blood samples at 1, 3, 6, 12, 24, and 36 h were also collected through the cannula. The rat serum was tested for BChE activity.

Five male, strain 129Sv mice, weighing 23 to 26 g and 55 days of age, were injected intraperitoneally with recombinant human BChE. A maximum of 0.2 ml of BChE, containing 144 units of butyrylthiocholine hydrolyzing activity, was injected i.p. for a dose of 9 mg/kg. The hair was shaved off a hind leg with a scalpel, and the saphenous vein was punctured with a needle. About 10 to 50 μl of blood was collected into a capillary tube at 0, 10, 30, 60, and 90 min, and 3, 5, 7, 22, 30, 56, and 96 h, and transferred to a microcentrifuge tube. The serum was tested for BChE activity. Similarly, 0.2 ml of purified native human BChE containing 438 units was injected i.p. into five mice, at a dose of 27 mg/kg. Blood samples were collected at 0, 1, 2, 4, 6, 8, 10, 24, 34, 48, 72, 96, 168, and 216 h.

The data for elimination of BChE from the circulation were fitted to a double-exponential equation in Sigma Plot (Jandel Scientific, Chicago, IL). The equation is described by Kronman et al. (2000) (see legend to Table 3) as well as by Saxena et al. (1998).

Locomotor Activity. A motion detector was made by the Instrument Shop at the University of Nebraska Medical Center. It consisted of a red-light-emitting diode that sent a beam of light through the cage wall, a photodiode detector on the opposite side of the cage, and a microprocessor. When the mouse passed through the beam, an event was recorded by the microprocessor. After a defined time, the total number of events was printed. The beam was set up in a dimly lit box containing the home cage in a sound-proof room. The cage contained no bedding because kicked-up bedding could trigger the beam counter. The mouse was acclimated for 1 h before beam breaks were counted.

Adult, male, strain 129Sv mice, 52 to 94 days of age, weighing 24 to 35 g and averaging 28.2 ± 3.0 g, were from a colony at the University of Nebraska Medical Center. Mice received either cocaine alone, saline alone, or BChE followed 1 h later by cocaine. Each mouse received cocaine only once. The dose of cocaine was 25 mg/kg i.p. The dose of BChE was 2.8 units/g i.p., where units were defined as micromoles of butyrylthiocholine hydrolyzed per minute. Animals were not returned to their home cage until at least 16 h after cocaine dosing because cocaine-treated animals displayed aggressive behavior for several hours after treatment.

Results

Catalytic Activity of A328W. The A328W mutant of BChE was tested for activity with (−)-cocaine, butyrylthiocholine, and benzoylcholine. Table 1 shows that A328W hydrolyzed (−)-cocaine 15-fold faster than did wild-type BChE as measured by k_{cat}. The binding affinity was nearly the same for both enzymes, the K_m value being 10 μM for A328W and 7 μM for wild-type BChE. Both enzymes hydrolyzed butyrylthiocholine and benzoylcholine much more rapidly than cocaine. However, the k_{cat} value for A328W was lower for these substrates compared with the k_{cat} for wild-type BChE.

Tetramers of Recombinant BChE. When BChE was expressed in stable CHO cell lines in the absence of the peptide from COLQ, the BChE consisted predominantly of dimers and monomers, as demonstrated on nondenaturing gel stained for BChE activity. Only 10% of the BChE activity was a tetramer (Blong et al., 1997; Altamirano and Lockridge, 1999). However, coexpression of 45 residues of the N terminus of the rat collagen-tail, called rQ45, resulted in 70% BChE tetramers (Fig. 2).

Elimination Half-Life in Rats. When a purified preparation of recombinant human BChE monomers and dimers was injected into the jugular vein of rats, the half-life was 2 min (Fig. 3), a result in agreement with that of Saxena et al. (1998). No detectable human BChE was present in rat serum after 1 h. In contrast, when a preparation containing 70% tetramers (wild-type BChE rQ45 or A328W rQ45) was injected into rats, the elimination was biphasic, with 81% disappearing with a half-life of 26 ± 3 min and 19% disappearing with a half-life of about 16 ± 6 h (Fig. 3). The mean residence time was 1245 min. Significant amounts of human BChE activity were still present in the rat 24 h after i.v. injection of tetrameric BChE. The 1 to 2.8 U/ml of BChE activity found after 24 h is a 40- to 50-fold increase above the endogenous BChE activity of 0.02 to 0.07 U/ml in rat serum.

The nondenaturing gel in Fig. 4 shows that tetramers of BChE remained in the circulation longer than monomers and dimers. Thus, the human BChE remaining after 24 h was essentially all tetrameric. This result is consistent with the results in Fig. 3, which showed rapid clearance of monomers and dimers of BChE. The lane labeled "0 min" contains 2 μl of rat serum taken before the rat was injected with human BChE. No bands showed up in this lane, supporting the finding that rat serum contains very little endogenous BChE activity.

The C2 band in Figs. 2 and 4 is a dimer formed by covalent bonding of one subunit of BChE and one of albumin (Masson, 1989). Purified BChE does not contain C2. Human serum contains C2. The C2 band in rat serum appeared only after the purified human BChE had been injected into the rat.

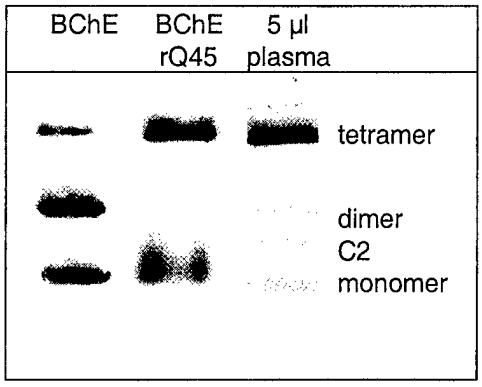

Fig. 2. Nondenaturing gel stained for BChE activity. Recombinant human BChE secreted from CHO cells consists mainly of monomers and dimers. Recombinant human BChE rQ45 consists of 70% tetramers. Human plasma contains more than 95% tetramers. The plasma is a control to show the migration of monomers, dimers, and tetramers of BChE. The C2 band is a dimer made by disulfide bonding of albumin and BChE (Masson, 1989).

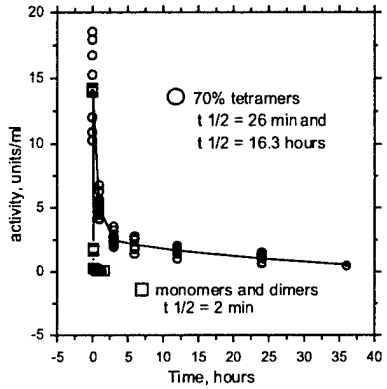

Fig. 3. Elimination half-life of recombinant BChE injected i.v. into rats. Purified recombinant wild-type BChE consisting of either 70% tetramers or 90 to 95% monomers and dimers was injected intravenously into rats (n = 5) at a dose of 1 mg/kg (250 units per rat). The same results were obtained with recombinant A328W rQ45.

Peak Activity in Blood. Five mice received intraperitoneal injections of the same purified BChE preparation (recombinant wild-type human BChE rQ45 containing 70% tetramers) that had been given to rats. Figure 5 shows that the human BChE rapidly entered mouse blood, where peak activity was achieved about 1 h after injection into the peritoneal cavity. Peak activity in mouse blood was 45-fold above the endogenous activity of 1 to 1.4 U/ml. The activity remained high for hours. After 7 h, it was still 20-fold over endogenous activity. Hoffman et al. (1996) had previously found that native human BChE injected i.p. reached peak activity in mouse blood 1 h after injection. Based on these results, we decided to allow 1 h to elapse between i.p. injec-

TABLE 1
Catalytic activity of wild-type BChE rQ45 and A328W rQ45 at 25°C in 0.1 M potassium phosphate, pH 7.0

Substrate	Wild-Type BChE		A328W	
	K_m	k_{cat}	K_m	k_{cat}
	μM	min^{-1}	μM	min^{-1}
(−)-Cocaine	7 ± 0.3	2 ± 0.2	10 ± 1	30 ± 3
Butyrylthiocholine	20 ± 2	30,000 ± 3,000	20 ± 2	24,000 ± 2,000
Benzoylcholine	5 ± 1	15,000 ± 1,000	5 ± 1	9,000 ± 1,000

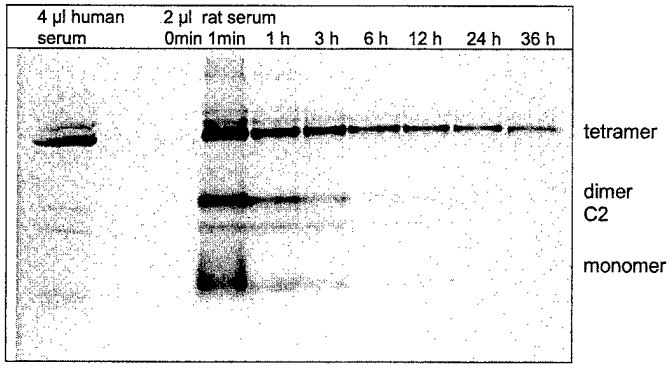

Fig. 4. Nondenaturing gradient polyacrylamide gel stained for BChE activity in rat. Blood samples were removed at various times after treatment of a rat with purified recombinant wild-type human BChE rQ45. Two microliters of serum were placed in each well of a 4 to 30% gradient polyacrylamide gel and electrophoresed, as described under *Materials and Methods*. Tetramers of human BChE were still present after 24 to 36 h, although dimers and monomers had disappeared. Human serum provides markers for tetramers, dimers, and monomers.

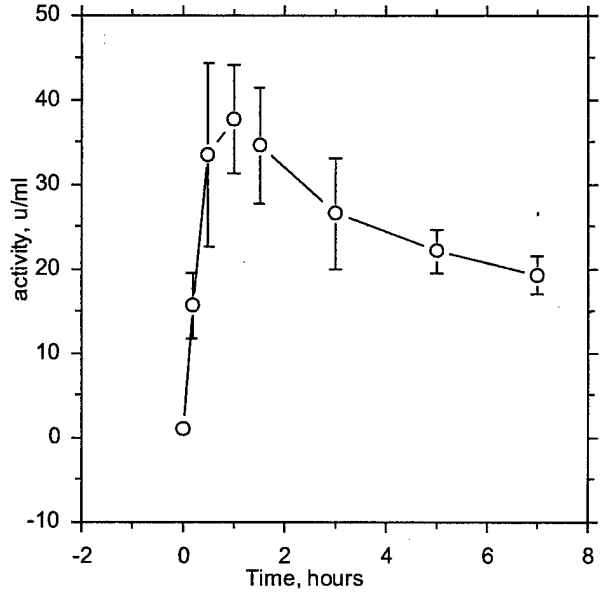

Fig. 5. BChE activity in mouse blood after intraperitoneal injection. Purified wild-type BChE rQ45 consisting of 70% tetramers was injected intraperitoneally into mice (n = 5) at a dose of 9 mg/kg.

tion of BChE and injection of cocaine in locomotor activity assays.

Elimination Half-Life in Mice for Recombinant BChE. Figure 6 shows that recombinant BChE disappeared from mouse blood in a biphasic manner, with 57% disappearing with a half-life of 48 ± 20 min and 43% with a half-life of 15.6 ± 2 h. The mean residence time was 1269 min. The pharmacokinetics of the purified human BChE rQ45 were similar in rats and mice.

Blood samples, taken from the mouse various times after i.p. injection of human BChE rQ45, were subjected to gel electrophoresis on a nondenaturing gradient gel. Each lane in Fig. 7 received 2 µl of mouse serum. The gel shows that all sizes of human BChE entered mouse blood from the peritoneum. Comparison of lanes 0 and 10 min shows that the human BChE bands migrated more slowly on the gel than the corresponding mouse bands, so that a doublet of tetramer bands is visible. Mouse BChE has 574 amino acids and 7

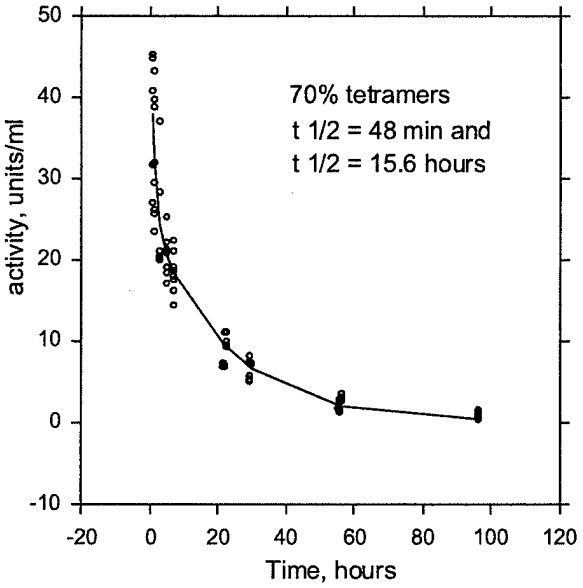

Fig. 6. Elimination half-life of recombinant human BChE injected i.p. into mice. Purified wild-type human BChE rQ45 consisting of 70% tetramers was injected intraperitoneally into mice (n = 5) at a dose of 9 mg/kg.

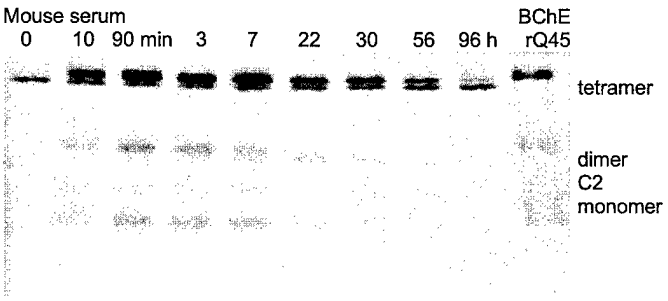

Fig. 7. Nondenaturing polyacrylamide gel stained for BChE activity in mouse. Blood samples were removed from a mouse that was treated intraperitoneally with purified recombinant wild-type human BChE rQ45. Two microliters of mouse serum were placed in each well.

glycan chains, whereas human BChE has 574 amino acids and 9 glycan chains. The faster migration of mouse BChE is explained by its lower molecular weight. Tetramers of human BChE remained in the mouse blood longer than dimers and monomers, a result similar to that observed in rat blood. The last lane of this gel shows the pattern of bands in the recombinant human BChE rQ45 before it was injected into the mouse. The monomer band is broader, suggesting that some of the diffuse forms did not enter the mouse blood. Another point to notice is that the purified human BChE rQ45 has no C2 band. The C2 band forms in the mouse probably by combining mouse albumin with one subunit of human BChE.

Elimination Half-Life in Mice for Native Human BChE. Figure 8 shows the BChE activity in mouse blood after an i.p. injection of 438 units of native human BChE tetramers (0.6 mg of BChE per mouse). High amounts of human BChE were present in mouse blood 1 to 10 h after injection. The mean residence time was 56.6 h. These results show that the residence time of native human BChE in the mouse circulation is 2.7-fold longer than the residence time of recombinant human BChE.

Cocaine Toxicity Measured as Locomotor Activity. A dose of 25 mg/kg cocaine i.p. caused increased locomotor

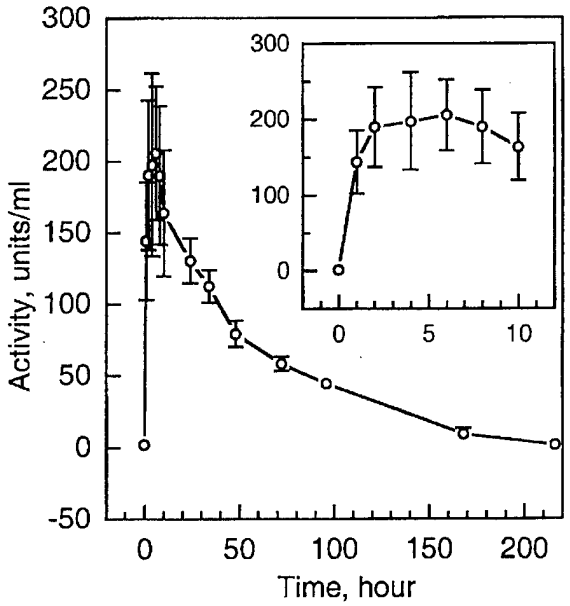

Fig. 8. Elimination half-life of native human BChE injected i.p. into mice. Purified native human BChE consisting of 99% tetramers was injected intraperitoneally into mice (n = 5) at a dose of 27 mg/kg.

activity in mice. Their behavior was markedly different from that of control mice (n = 32) injected with saline or BChE alone, who ambled back and forth for a few minutes, sniffed, defecated, urinated, then settled down and went to sleep (Fig. 9). Locomotor hyperactivity was therefore taken to be a good indicator of cocaine toxicity.

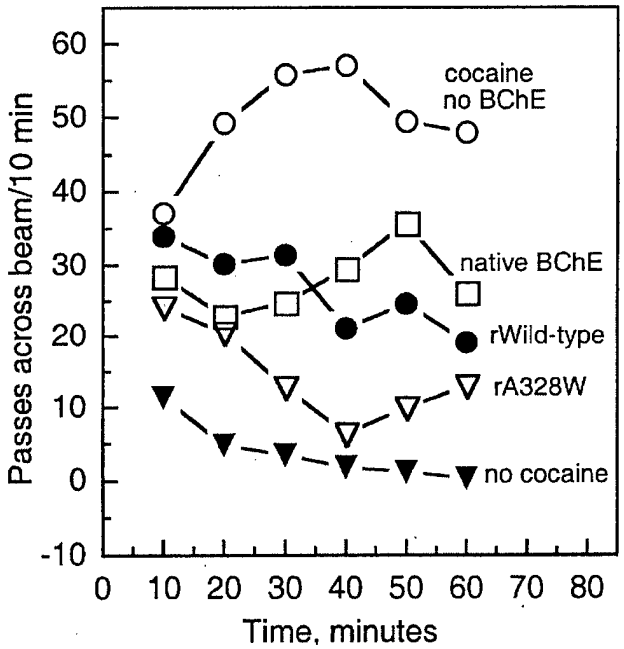

Fig. 9. Locomotor activity in mice as a measure of cocaine toxicity. Mice were acclimated for 1 h before receiving 25 mg/kg cocaine i.p. (n = 10). Another group of mice (n = 10) was acclimated for 1 h, then received 2.8 units/g of wild-type BChE rQ45 i.p., followed in 60 min by an injection of 25 mg/kg cocaine i.p. A third group (n = 10) was pretreated with 2.8 units/g of A328W rQ45 before receiving cocaine. A fourth group (n = 15) was pretreated with 2.8 units/g of native human BChE before receiving cocaine. Locomotor activity was recorded for 60 min following BChE injection and 60 min after cocaine injection. The A328W mutant of BChE was more effective than wild-type BChE at attenuating locomotor activity induced by cocaine.

The protective effect of human BChE against cocaine toxicity was tested by pretreating mice with either recombinant wild-type BChE rQ45 (n = 10) or A328W rQ45 (n = 10) or native wild-type BChE (n = 15) at a dose of 2.8 units/g (units measured with 1 mM butyrylthiocholine). One hour after i.p. injection of BChE, the mice were injected i.p. with cocaine at a dose of 25 mg/kg.

Figure 9 shows the number of beam breaks per 10 min as a function of time after injection of cocaine. Mice that received cocaine alone (no BChE) were hyperactive immediately after injection of cocaine; their activity increased during the first 10 min and remained high for the next 60 min. Mice pretreated with recombinant wild-type BChE, or with native wild-type BChE, were 50% less active, and mice pretreated with recombinant A328W were 80% less active (Table 2). These results indicate that both wild-type BChE and A328W have a protective effect against cocaine.

In the locomotor experiments by Mattes et al. (1997), the same rats were retested repeatedly with cocaine. This strategy did not work for us. A single injection of cocaine made mice supersensitive to cocaine for as long as 2 weeks. This phenomenon of sensitization is known in the literature (Carey and Gui, 1998). To avoid the complications of sensitization to cocaine, we used a naive mouse for each experiment.

Discussion

Protection against Cocaine Toxicity. Both wild-type recombinant human BChE and the A328W mutant BChE protected mice from cocaine-induced hyperactivity. The A328W mutant gave more protection, consistent with its higher catalytic activity toward cocaine. Native human wild-type BChE, purified from human plasma, gave a level of protection similar to that of recombinant wild-type human BChE.

Our results with recombinant wild-type BChE agree with the results of others who had used native BChE. Mattes et al. (1997), using native human BChE, and Carmona et al. (1998), using horse BChE, found that pretreatment with BChE attenuated cocaine-induced locomotor activity in rats.

A 22-g mouse contains a total of 17 units of BChE activity (0.3 nmol) in its body, 60% in the intestine and liver, and 20% in plasma (Duysen et al., 2001). The dose of human BChE in the protection experiments was 2.8 units/g which for a 22-g

TABLE 2

Effect of BChE pretreatment on cocaine-induced locomotor activity in mice

Statistical significance was evaluated by analysis of variance using Bonferroni correction to adjust for multiple comparisons.

Treatment	Number of Beam Breaks in 60 min
Saline alone (n = 2)	145 ± 54
BChE alone 2.8 units/g i.p. (n = 30)	129 ± 99
Cocaine 25 mg/kg i.p. (n = 10)	1463 ± 414^{abc}
Native human BChE 2.8 units/g i.p.; cocaine 25 mg/kg (n = 15)	643 ± 343^{a}
Recombinant wild-type BChE 2.8 units/g i.p.; cocaine 25 mg/kg (n = 10)	797 ± 342^{bd}
Recombinant A328W BChE 2.8 units/g i.p.; cocaine 25 mg/kg (n = 10)	420 ± 85^{cd}

$^{a-d}$ The two numbers labeled with superscript a were significantly different with a probability value of $p \leq 3.6 \times 10^{-5}$; b were different with probability $p \leq 6.5 \times 10^{-7}$; c were different with probability $p \leq 0.0018$; and d were different with probability $p \leq 0.006$.

mouse is 3-fold over endogenous BChE content. If the same dose of BChE is required to protect a human, about 270 mg of purified BChE will be needed to protect a 70-kg person.

Recombinant BChE with a 16-h Half-Life in the Circulation. This is the first report in which recombinant human BChE has been used in protection experiments. Previous work has used native BChE purified from plasma for protection against cocaine toxicity (Hoffman et al., 1996; Lynch et al., 1997; Mattes et al., 1997; Carmona et al., 1998) and for protection against organophosphorus toxicants (Broomfield et al., 1991; Doctor et al., 1991; Brandeis et al., 1993; Raveh et al., 1997).

The problem of short residence time in the circulation of animals had to be solved before recombinant BChE could be studied for its protective properties. Monomers and dimers of recombinant human BChE disappeared within minutes from the circulation of mice (Saxena et al., 1998). On the other hand, native human serum BChE is a tetramer, and its half-life in the circulation is 7 to 12 days in humans, or 2 days in the circulation of mice. We therefore aimed to make BChE tetramers. The breakthrough that made it possible to produce recombinant BChE tetramers was the finding by the laboratory of Jean Massoulié in Paris that a 17-residue proline-rich peptide was required for assembly into tetramers. Bon et al. (1997) reported that assembly of rat AChE into tetramers required the N-terminal peptide from the collagen-tail. Since AChE and BChE are homologous in many features, it was reasonable to expect that this same collagen-tail peptide would facilitate assembly of BChE as well. When the collagen-tail peptide was coexpressed with BChE in CHO cells, the resulting BChE was a tetramer (Altamirano and Lockridge, 1999). Without the collagen-tail peptide, the recombinant BChE consisted mainly of monomers and dimers.

The half-life of recombinant human BChE tetramers was about 16 h in rats and mice. This is an improvement of 480-fold over the half-life of BChE monomers and dimers, which were cleared from the circulation within 2 min. Native human BChE had a 2.7-fold longer residence time in the circulation of mice than recombinant BChE.

Carbohydrate Content of Recombinant BChE. Saxena et al. (1998) found that recombinant human BChE monomers and dimers secreted by CHO cells are underglycosylated. Recombinant BChE has only five *N*-glycans, whereas native human BChE has nine. The recombinant BChE had a ratio of sialic acid to galactose of about 1, suggesting that nearly all galactose residues were capped with sialic acid. Saxena et al. (1998) concluded that the capping of galactose with sialic acid by itself is not sufficient to confer circulatory stability and that high molecular weight is also important. Our results support the conclusion that the rapid clearance of BChE monomers and dimers is not due to incomplete sialylation but to their small size of 85 and 170 kDa. It was not necessary to modify the carbohydrate content of the recombinant BChE to attain a half-life of 16 h. Assembly into tetramers achieved the desired goal. This contrasts with recombinant AChE expressed in human embryonic kidney 293 cells (Kronman et al., 2000), where a half-life of 15 h was obtained only after modification of the carbohydrate content and after assembly into tetramers. The importance of large molecular size was confirmed by Cohen et al. (2001), who attached polyethylene glycol to AChE monomers and found that high molecular weight monomers had a half-life of 26 h in the circulation of mice, even though only 60% of the *N*-glycans were sialylated.

CHO Cells. CHO cells are the preferred cell type for expression of human glycoproteins of potential therapeutic value such as erythropoietin, human growth factor, and tissue plasminogen activator (Jenkins and Curling, 1994). Our decision to produce recombinant human BChE in CHO cells was made after we compared growth conditions and yield of BChE in CHO cells and in 293 human embryonic kidney cells. The 293 cells are used to produce human AChE (Kronman et al., 2000). We found that the yield of human BChE was similar in both cell lines. Both cell lines secreted human BChE to a maximum of 5 mg/l. Both cell lines produced monomers and dimers and very few tetramers. Kronman et al. (2000) had to modify the 293 cells to produce α2,6-sialyltransferase, an enzyme deficient in 293 cells, to fully sialylate the AChE. Although CHO cells are also deficient in α2,6-sialyltransferase (Jenkins and Curling, 1994), the recombinant BChE produced by CHO cells was fully sialylated (Saxena et al., 1998), and the tetrameric BChE produced by CHO cells had a half-life of 16 h without introducing sialyltransferase. An additional advantage of CHO cells is their ability to grow in the absence of fetal bovine serum.

Purification of Recombinant BChE. Recombinant BChE secreted by CHO cells was collected over a period of months. The culture medium was stored at 4°C until 100 mg of BChE had accumulated. No loss of BChE activity resulted during the storage period. A two-step purification procedure consisting of affinity chromatography on procainamide-Sepharose followed by ion-exchange chromatography on DE52 resulted in highly purified BChE tetramers. The simplicity of the purification protocol makes it possible to produce gram quantities of highly purified recombinant BChE.

Potential for Use of Human BChE in People. Purified BChE has a history of use in the clinic. The literature reports 134 patients who received partially purified human BChE for treatment of prolonged neuromuscular block by the muscle relaxants succinylcholine and mivacurium or for treatment of organophosphorus pesticide poisoning (Table 3). The BChE injected into these patients was a 5% pure preparation from human plasma sold by Behringwerke (Marburg, Germany). It is a dry concentrate of "cholase". The patients recovered and had no side-effects from the BChE treatment.

TABLE 3
Clinical use of purified human BChE
Patients were treated for succinylcholine or mivacurium apnea, or for toxicity from organophosphorus pesticide.

Number of Patients	Reason for using BChE	Reference
4	Succinylcholine	Evans et al. (1953)
23	Succinylcholine	Borders et al. (1955)
1	Parathion	Goedde and Altland (1971)
1	Succinylcholine	Stovener and Stadskleiv (1976)
1	Demeton-*o*-methyl	Klose and Gutensohn (1976)
12	Succinylcholine	Scholler et al. (1977)
1	Succinylcholine	Schuh (1977)
10	Succinylcholine	Viby-Mogensen (1981)
1	Parathion	Cascio et al. (1988)
1	Succinylcholine	Benzer et al. (1992)
2	Mivacurium	Ostergaard et al. (1995)
1	Mivacurium	Naguib et al. (1995b)
40	Mivacurium	Naguib et al. (1995a)
20	Mivacurium	Naguib et al. (1996a)
16	Mivacurium	Naguib et al. (1996b)

All of the reports in Table 3 are from Europe or Saudi Arabia, as human BChE is not yet approved for human use in the United States.

In conclusion, human BChE can now be produced in gram quantities by expression in CHO cells. The recombinant BChE consists predominantly of tetramers, and these have an elimination half-life of 16 h in rodents. Both wild-type and A328W mutant human BChE have the potential to be clinically useful for treating cocaine toxicity in humans.

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Research report

Rescue of the acetylcholinesterase knockout mouse by feeding a liquid diet; phenotype of the adult acetylcholinesterase deficient mouse

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Abstract

Acetylcholinesterase (AChE, EC3.1.1.7) functions in nerve impulse transmission, and possibly as a cell adhesion factor during neurite outgrowth. These functions predicted that a mouse with zero AChE activity would be unable to live. It was a surprise to find that AChE −/− mice were born alive and survived an average of 14 days. The emaciated appearance of AChE −/− mice suggested an inability to obtain sufficient nutrition and experiments were undertaken to increase caloric intake. Pregnant and lactating dams (+/−) were fed 11% high fat chow supplemented with liquid Ensure®. AChE −/− pups were weaned early, on day 15, and fed liquid Ensure. Although nullizygous animals showed slow but steady weight gain with survival over 1 year (average 100 days), they remained small at all ages compared to littermates. They demonstrated delays in temperature regulation (day 22 vs. 15), eye opening (day 13 vs. 12), righting reflex (day 18 vs. 12), descent of testes (week 7–8 vs. 4), and estrous (week 15–16 vs. 6–7). Significant physical findings in adult AChE −/− mice included body tremors, abnormal gait and posture, absent grip strength, inability to eat solid food, pinpoint pupils, decreased pain response, vocalization, and early death caused by seizures or gastrointestinal tract ileus. Behavioral deficits included urination and defecation in the nest, lack of aggression, reduced pain perception, and sexual dysfunction. These findings support the classical role for AChE in nerve impulse conduction and further suggest that AChE is essential for timely physical development and higher brain function.

Theme: Development and regeneration

Topic: Neurotransmitter systems and channels

Keywords: Postnatal development; Gene targeting; Under-nutrition; Acetylcholinesterase depletion

1. Introduction

Acetylcholinesterase has a central role in neurotransmission at cholinergic synapses. AChE hydrolyzes acetylcholine, thus preventing overstimulation of nicotinic and muscarinic receptors. Overstimulation of these receptors may lead to respiratory failure and death. AChE has been proposed to have a second function independent of its catalytic activity [1–4,9,11,13–16,23,25,26,28,30–32], possibly serving as a cell adhesion factor in morphogenesis of neurites. These important roles for AChE predicted that the absence of AChE activity in mutant mice would be lethal. It was a surprise, therefore, to find that mice with no AChE enzyme activity and no AChE protein were born alive, and that they were capable of breathing and moving [35]. They were not normal, however. Homozygous mutant mice were smaller than heterozygous littermates, they gained body weight more slowly, their eyes never opened, they had no righting reflex, the external ear did not mature, body tremor was persistent, they circled when walking, and they died at an early age. About 50% of the nullizygotes died by postnatal day 14 and 100% died by day 21 (n=63). This phenotype could be due to deficiency in cholinergic neurotransmission caused by absence of AChE enzyme activity, or to developmental problems caused by

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the absence of the AChE protein, or to a combination of both.

In the present work our goal was to prolong the life of AChE −/− mice. Injection of purified AChE or of atropine had no beneficial effect, so we tried a different approach. Their emaciated appearance and absence of body fat suggested that the cause of death might be starvation. Therefore, efforts were made to increase their caloric intake. This report describes the success of feeding dams a high fat diet supplemented with liquid Ensure® to enrich their milk during the nursing period. After weaning, the AChE −/− mice subsist on liquid Ensure. This diet increased the lifespan of AChE −/− mice to an average of 100 days. Several AChE −/− mice have lived up to 15 months.

Having succeeded in producing an abundant supply of AChE −/− mice, we were able to investigate which characteristics were due to deficiency of AChE catalytic activity. Since pinpoint pupils, body tremor, and muscle weakness are present in AChE −/− mice and are also diagnostic for poisoning by AChE inhibitors, we concluded that these abnormalities were due to lack of AChE catalytic function. However, other abnormalities, including postnatal developmental delay, low body weight, lack of housekeeping behavior, and sexual dysfunction have no obvious link to absence of AChE catalytic function and could be due to a combination of effects.

2. Materials and methods

2.1. Mice

Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the US National Institutes of Health. AChE knockout mice were produced by homologous recombination [34,35]. Exons 2, 3, 4, and 5 of the ACHE gene were deleted, making it impossible to produce an AChE protein. The colony was maintained by breeding AChE +/− mice. The animals are in a strain 129Sv genetic background, produced by mating the chimera (originating from R1 embryonic stem cells) to strain 129Sv mice (Taconic 129S6/SvEvTac). Mice are housed in a barrier facility at a temperature of 66–74 °F, humidity of 40–42%. Lights are on for 12 h a day.

2.2. Genotyping mice

Mice were genotyped by PCR. The preparation of genomic DNA and the PCR primers were different from those published earlier [35]. Genomic DNA was isolated from the hair roots of mice 14 days of age or older, by the method of Schmitteckert et al. [27]. A tuft of hair roots was placed in a 0.5-ml microfuge tube. After the addition of 50 μl of 0.05 M NaOH, the tubes were heated at 95 °C for 5 min in the thermocycler, and then cooled to 4 °C. The DNA concentration, estimated from absorbance at 260 nm, ranged from 0.15 to 1.4 μg/μl depending on the number of hair roots. A 10-μl PCR reaction was set up to contain a total of 0.35 μg DNA, 0.25 units of HotStar Taq Polymerase (Qiagen Inc., Valencia, CA), 0.2 mM of each dNTP, 0.02 μg of each primer, and 1 μl of PCRx Enhance Solution (Gibco-BRL #52391). Primers for wild-type ACHE were 21mer sense (5′AATGACACCGAGCTGATAGCC) and 22mer antisense (5′CCAGTATTGATGAGAGCCTCCG), both located within exon 2 of ACHE. Primers for the ACHE knockout allele were 25mer sense (5′AATGGGCAGGTAGCCGGATCAAGCG) in the NEO gene and 25mer antisense (5′CGTAGTCTCGTCGGCTAACAGACAA) also in the NEO gene. The wild-type allele produced a band of 164 bp, whereas the knockout allele had a band of 322 bp. HotStar Taq Polymerase was activated by heating at 95 °C for 15 min. The DNA was denatured at 94 °C for 1 min 15 s, annealed at 60 °C for 1 min 30 s, and extended at 72 °C for 1 min 30 s in 35 cycles on a Perkin Elmer Cetus DNA Thermal Cycler.

Newborn mice do not have hair. Therefore, tail snips were the source of DNA for genotyping mice younger than 14 days. DNA was extracted from tail snips using QIAamp DNA Mini Kit (catalog number 51306). The total amount of DNA in a 10-μl PCR was 0.2 μg.

2.3. Food for mice

Solid food pellets were purchased from Harlan Teklad, Madison, WI. Harlan Teklad LM-485 Irradiated Mouse Sterilizable Diet 7012, catalog #7912, contains 5% fat. Harlan Teklad S-2335 Irradiated Mouse Breeder Sterilizable Diet 7004, catalog #7904, contains 11% fat. A list of components of the Harlan Teklad diets can be found at http://www.harlan.com. Liquid food was purchased from a local grocery or drugstore, and later, 30 cases at a time (24×8-oz cans per case) from Abbott Laboratories (1-800-222-6883). The liquid food is manufactured by Abbott Laboratories, Ross Products Division, Columbus, OH. Ensure Light®, vanilla flavor, catalog # 52770; Ensure Plus®, vanilla flavor, catalog # 50464; Ensure Fiber with FOS®, vanilla flavor, catalog #50650.

Ensure is a complete food for a human being, containing all essential vitamins, minerals, folic acid, choline, and other essential nutrients. Ensure Plus has the highest number of calories and the highest fat content, at 360 calories and 11 g fat per 8 fl oz (236.5 ml). Ensure Fiber has 250 calories and 6 g fat per 236.5 ml; in addition, it has 4 g of fiber. Ensure Light has 200 calories and 3 g fat per 236.5 ml. The ingredients in each type of Ensure are described at http://RPDIND01.ROSS.COM/-85256404006C18D6/.

2.4. Containers for liquid food

Liquid food was fed to lactating dams through a glass bottle (20-ml glass scintillation vial) fitted with a one-hole rubber stopper and glass tube. The bottle was inserted into the wire cage top so that the glass tube protruded into the cage. An alternative method, that of suspending the bottle inside the cage from a bottle hanger, was unsatisfactory because many dams buried the bottle with bedding. Since nullizygotes were unable to eat solid food or lift their heads to drink from a suspended bottle, liquid food for weaned nullizygotes was placed in a small Petri dish on top of paper towels. Paper towels were used as bedding because standard bedding was kicked into the Petri dishes, soaking up the food. Paper towels were replaced daily. Food containers were washed daily.

2.5. Body temperature maintenance

One to three nullizygotes were housed in standard plastic mouse cages with a wire top. Alternatively, up to eight nullizygotes were housed in a standard hamster cage. Two plastic box tops (top of a box of 200 μl pipette tips, 12×8.3×3.2 cm) were inverted and placed inside the hamster cages to provide warmth and a place to hide. A hole was cut into one side of each plastic box top to allow entry, and the boxes were lined with paper towels. Most nullizygotes were incapable of making a nest; therefore nesting material was not given to nullizygotes. Nullizygotes between the ages of 15 and 22 days required additional measures to stay warm. They were placed in cages that rested partially on an 11×13 inch heating pad (purchased from Osco Drugs), providing a temperature of 42.3 °C at that end of the cage floor.

2.6. Thermometer

The axial body temperature of mice was measured with a digital thermometer, Thermalert model TH-5 and a surface Microprobe MT-D, Type T thermocouple (Physitemp Instruments Inc., Clifton, NJ). A surface probe was used because very young mice are too small to allow the use of an anal probe. AChE −/− mice were handled every day for measurement of temperature and weight. A drop in temperature and body weight was an indicator of failure to thrive.

2.7. Grip strength

The device for measuring grip strength consisted of a screen mounted on a rod that could be rotated 180°. The mouse was placed on top of the screen, the screen was rotated 180°, and the time it took the mouse to fall off was measured. If the mouse could cling to the screen for 60 s or climbed to the top of the screen, it was scored as having normal grip strength.

2.8. Footprints

Feet were dipped in nontoxic paint and footprints recorded on white paper. Different colors were used for front and back feet.

2.9. Locomotor activity

A motion detector was made by the Instrument Shop at the University of Nebraska Medical Center. It consisted of a red-light-emitting diode which sent a beam of light through the cage wall, a photodiode detector on the opposite side of the cage, and a microprocessor. When the mouse passed through the beam, an event was recorded by the microprocessor. After a defined time, the total number of events was printed. The beam was set up in a sound proof, dimly lit box containing the home cage. The mouse was acclimated for 24 h before beam breaks were counted.

2.10. Neurobehavioral observations

The righting reflex was used to test motor and balance functions. Mice were placed on their backs on a piece of cardboard, giving them a rough surface for better grip. The time it took to return to an upright position was recorded. The tail pinch test was used to measure pain response. Reactivity to a light pinch with metal forceps was observed. Ability to correct orientation was measured in the geotaxis test. Mice were placed on a 45° inclined plane head down, an unnatural position for mice. The mice were observed for the ability to turn around with the head facing up the incline. Balance and hind limb strength were tested by counting the number of times each mouse reared after being placed in a novel environment, that is, displaying a bipedal posture with front feet off the ground. Pupil response was measured by shining a light into the eyes of the mice and observing constriction of the pupils. Resistance to restraint was measured as resistance to being held. Tests were from McDaniel and Moser [20].

2.10. Olfaction

The ability to smell was measured by placing 0.05 g of peanut butter in one corner of a hamster cage. A mouse was placed in the diagonal corner of the cage 46 cm away. The time it took the mouse to reach the peanut butter and actively sniff it from a distance of less than 1 cm was measured.

2.11. Blood chemistry

Whole blood was collected into serum separation tubes. Serum was analyzed for listed analytes by the Omaha Animal Medical Group, Omaha, NE.

3. Results

3.1. Husbandry

3.1.1. High fat diet for dams

Food for pups consists mainly of milk on days 1–21. Therefore, the milk was enriched by feeding dams a high fat diet. Female mice had been fed 5% fat chow for 10–12 days during the time they were housed with a male mouse. Immediately after the breeding period, female mice were fed 11% fat chow. They continued to receive 11% fat food pellets during pregnancy and while pups were nursing. The diet of lactating dams was supplemented with liquid Ensure, offered in a 20-ml bottle. This strategy, in combination with hand feeding, was successful as judged by the survival of 97% of AChE −/− pups to the day of weaning (n=100).

3.1.2. Hand feeding and weaning

Starting on postnatal day 12 and continuing until the day of weaning, all AChE −/− pups were hand fed twice a day. The pup was held by the scruff of the neck and drops of Ensure, delivered with a pipette tip, were placed into its mouth. The purpose of hand feeding with Ensure was twofold. Firstly, this diet supplement increased their chance of surviving, and secondly, they became familiar with the taste of Ensure. After weaning, their food consisted entirely of Ensure, so it was necessary to acclimate them to this food, to be sure they would eat it.

Though the normal age for weaning is postnatal day 21, the AChE −/− mice were weaned earlier, on postnatal day 15. On day 15, AChE −/− mice were placed into a separate cage that rested partly on a heating pad. The AChE −/− mice required additional warmth because they were not able to regulate their temperature by day 15. A small plastic box (12×8.3×3.2 cm) lined with a paper towel was inverted and placed inside the cage to provide extra warmth. A second reason for weaning AChE −/− mice at this early age is that they need to have food available 24 h a day. They do not get enough nutrition through nursing and can starve to death if left with the dam to day 21. It was not feasible to provide AChE −/− mice with Ensure in the home cage because AChE −/− mice can only eat from an open dish and the Petri dish became covered with bedding.

3.1.3. Culling

Litters were culled to 5–6 pups on postnatal day 5 by removing normal pups. The AChE −/− pups were easily identified by their body tremor, which was especially noticeable when the pup was laid upside down. Culling resulted in larger body size for AChE −/− pups.

3.1.4. Body temperature

By day 15, AChE +/+ and +/− pups maintained a normal body temperature of 36.7 °C, whereas AChE −/− pups had an average temperature of 34 °C (Fig. 1). By day 15, the huddling time was decreased and the nesting material flattened. This caused the AChE −/− mice to become hypothermic. The ability to regulate body temperature was delayed by 7 days in AChE −/− mice, who reached a more normal body temperature on day 22. As shown in Fig. 1, even mature AChE −/− mice had lower body temperatures than wild-type mice. The maximum surface body temperature of AChE −/− mice averaged 36.5 °C, while that of AChE +/+ mice averaged 37.1 °C.

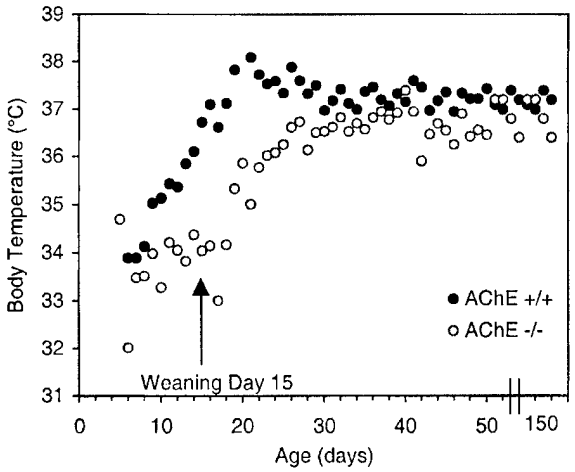

Fig. 1. Developmental delay in control of surface body temperature in AChE −/− mice. Temperature of AChE −/− mice (12 female+11 male) and AChE +/+ mice (13 female+13 male) was measured daily.

3.1.5. Food for AChE −/− mice after weaning

Since AChE −/− mice did not eat a substantial amount of solid food, liquid food was provided in the form of Ensure. There are a variety of Ensure formulations, differing in the amount of fat and calories per ml. Our initial observations had been made with Ensure Plus. To identify the Ensure that gave the highest survival rate, mice were fed three different Ensure formulations. Food consumption was measured over a period of 5 months and correlated with body weight and survival. Ensure was available to AChE −/− mice 24 h a day. Fresh Ensure was provided every day in clean Petri dishes.

Fig. 2 correlates body weight with the type of Ensure in the diet. Female AChE +/+ mice in Fig. 2A and male AChE +/+ mice in Fig. 2B responded with similar gain in body weight regardless of the type of Ensure. Female wild-type mice reached a stable weight of about 27 to 32 g by day 150, while male wild-type mice reached a weight of 30–35 g. The same pattern of weight gain was observed when AChE +/+ mice were fed the standard 5% fat pelleted chow. Similarly, the rate of weight gain for AChE −/− mice in Fig. 2C was independent of the type of Ensure. There was no difference in the rate of weight gain for male and female AChE −/− mice, so the results for both genders are grouped. Adult nullizygotes are small

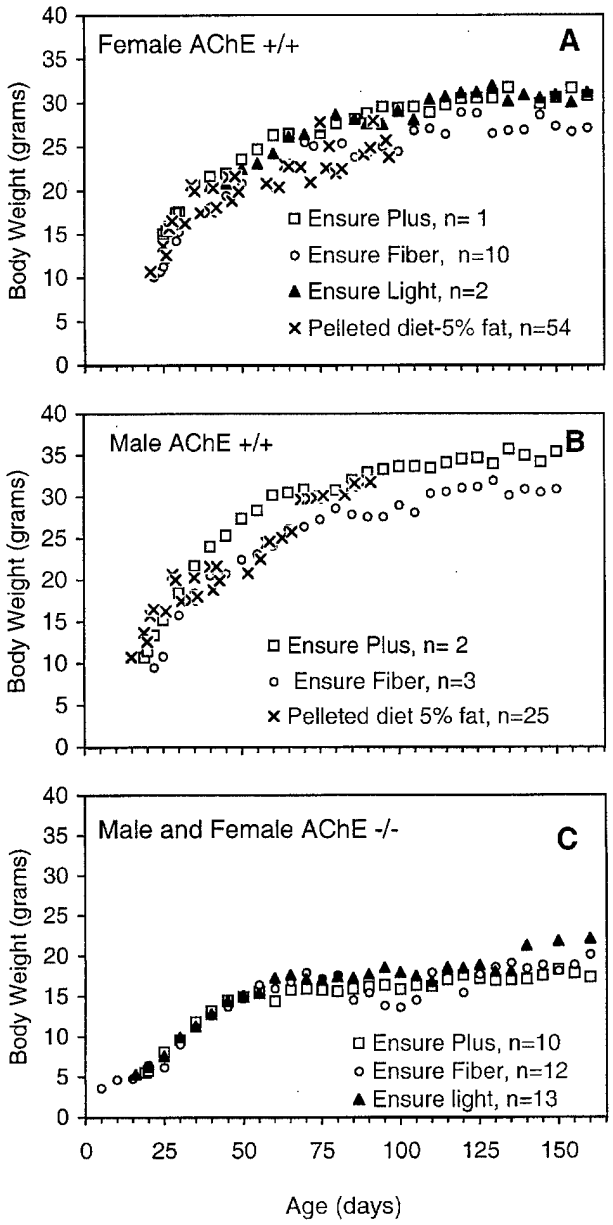

Fig. 2. Gain in body weight as a function of type of Ensure in the diet. Panel A, female AChE +/+ mice were fed Ensure Plus (n=1), Ensure Fiber (n=10), Ensure Light (n=2), or standard pelleted lab chow containing 5% fat (n=54). Panel B, male AChE +/+ mice were fed Ensure Plus (n=2), Ensure Fiber (n=3) or standard pelleted lab chow (n=25). Panel C, AChE −/− mice were fed Ensure Plus (4 female+6 male), Ensure Fiber (7 female+5 male), or Ensure Light (6 female+7 male).

relative to normal littermates, attaining an average body weight of about 18 g.

The rate of weight gain in AChE −/− mice was slower than in normal littermates. Fig. 3 shows that between days 6 and 14, there was either no gain in weight or there was loss of weight. This was a critical time for the AChE −/− mice. They had to be weaned to stop the weight loss and to avert death.

Survival of mice on different diets is compared in Fig. 4.

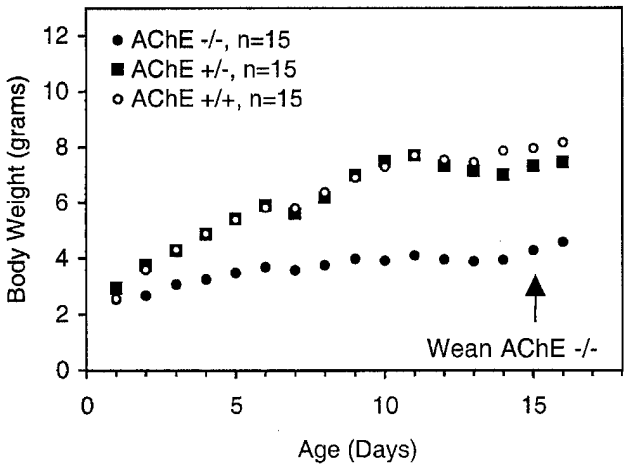

Fig. 3. AChE −/− neonates gain weight at a slower rate than wild-type and heterozygous mice. All mice were fed Ensure Fiber (6 female+9 male in each group).

All AChE +/+ mice lived longer than 250 days regardless of diet. By contrast, survival of AChE −/− mice depended on diet. The age for 50% survival of AChE −/− mice was 100 days on Ensure Fiber, 60 days on Ensure Plus, 60 days on Ensure Light, and 14 days in the absence of Ensure. Only one AChE −/− mouse from the group of 10 mice in the study has survived past 250 days, and that mouse is on Ensure Light. This mouse was euthanized at the age of 470 days. It was concluded that all three types of Ensure had similar beneficial effects, though Ensure Fiber gave a better average survival rate. Ensure Fiber had

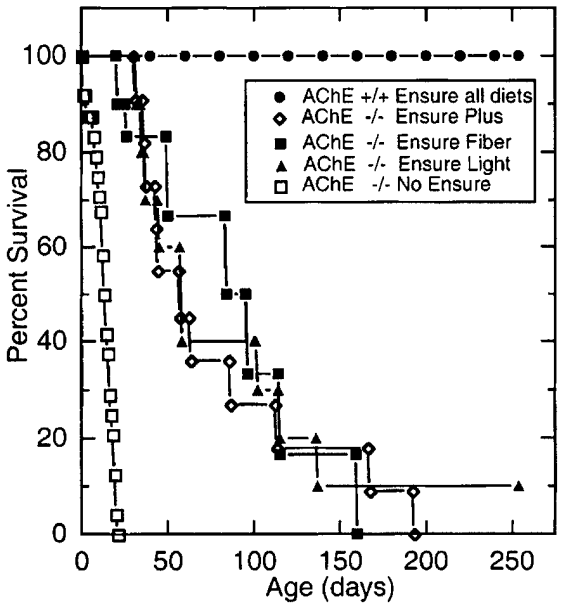

Fig. 4. Survival of AChE −/− mice depends on diet. AChE −/− mice were fed Ensure Plus (5 female+6 male), Ensure Fiber (5 female+4 male), Ensure Light (5 female+5 male), or no Ensure (11 female+13 male). All AChE +/+ mice (13 female+7 male) survived regardless of the type of Ensure they ate.

Table 1
Food consumed by wild-type (AChE +/+) and knockout (AChE −/−) mice. Calories consumed per gram of body weight per day

Age (days)	Ensure Fiber		Ensure Light		Ensure Plus	
	AChE +/+ (n=4)	AChE −/− (n=9)	AChE +/+ (n=3)	AChE −/− (n=10)	AChE +/+ (n=3)	AChE −/− (n=11)
16–40	0.86	1.01*	0.79	0.87	0.94	1.12*
41–80	0.60	0.73*	0.59	0.72*	0.60	0.74*
81–120	0.56	0.66*	0.44	0.67*	0.50	0.78*
121–160	0.53	0.70*	0.42	0.67*	0.48	0.80*

*Significantly different from AChE +/+ P=0.02 by single factor ANOVA.

the additional advantage of supplying fiber. Ensure Fiber was selected as the standard food for AChE −/− mice.

3.1.6. Fecal plug

AChE −/− mice between the ages of 15 and 22 days always chose the warmest spot in the cage, nesting in the area of the cage warmed by the heating pad. Mice on the heating pad often formed a fecal plug. Feces dried on the outside of the anus, blocking exodus of new feces. Mice were checked for the presence of fecal plugs daily. The fecal plug was softened with water and removed with tweezers. Voluminous soft feces flowed out after the plug was removed. Fecal plugs did not form after AChE −/− mice were removed from the heating pad.

For comparison, wild-type mice were separated from their mothers starting on postnatal day 13. Their housing was similar to that of AChE −/− mice. The food available to them was Ensure in a dish as well as solid food pellets on the cage floor. The 13–20-day-old wild-type mice also chose to nest in the area of the cage warmed by the heating pad. However, wild-type mice never developed a fecal plug.

3.1.7. Teeth

AChE −/− mice developed incisors at the same rate as their littermates. The presence of teeth rules out one possible explanation for their inability to eat solid food. They are capable of using their teeth for some gnawing, as evidenced by the shredding of paper towels. Incisors of normal mice remain worn down by chewing food pellets. Since the AChE −/− mice eat only liquid food, their front teeth can grow excessively long and become misshapen. In some mice, the teeth had to be trimmed every day to prevent penetration into the soft tissues of both the upper and lower jaw.

3.1.8. Calories consumed per gram of body weight

Food consumption was measured daily for 5 months by weighing the amount of Ensure consumed, and correcting for evaporation. Table 1 compares calories consumed per gram of body weight per day for AChE +/+ and −/− mice on three different diets. Younger animals consumed more calories per gram of body weight than older animals. Food consumption was similar for all three types of Ensure. The important difference between AChE +/+ and −/− mice was that AChE −/− mice consumed 20–50% more calories per gram of body weight per day at all ages. For example, adult AChE −/− mice in the age group 120–160 days consumed 0.70 calories per gram of body weight per day, whereas AChE +/+ mice consumed 0.53 calories per gram of body weight per day of Ensure Fiber. The requirement for more calories suggests that AChE −/− mice have a higher metabolic rate, or that they have decreased absorption of nutrients.

3.1.9. Blood chemistry

Blood analytes were tested to determine the physiologic status of AChE −/− mice. In semi-starvation and malnutrition the levels of glucose, total protein, phosphorus, albumin, cholesterol, and potassium may be low, while blood urea nitrogen is high [12]. Table 2 shows that blood

Table 2
Analysis of serum from adult mice

Component	AChE +/+	AChE −/−
Glucose, mg/dl	165.0 (S.D. 1.7) (n=2)	164.7 (S.D. 31.2) (n=5)
Total protein, g/dl	5.4 (S.D. 0.3) (n=2)	5.5 (S.D. 0.9) (n=4)
Blood urea nitrogen, mg/dl	20.1 (S.D. 3.4) (n=2)	17.5 (S.D. 4.9) (n=4)
Phosphorus, mg/dl	10.7 (S.D. 0.1) (n=2)	9.2 (S.D. 1.4) (n=3)
Albumin, g/dl	2.42 (S.D. 0.11) (n=2)	2.57 (S.D. 0.54) (n=4)
Cholesterol, mg/dl	109.9 (n=1)	106.0 (S.D. 10.5) (n=2)
Sodium, mmol/l	155.5 (S.D. 1.63) (n=2)	139.85 (S.D. 7.14) (n=2)
Potassium, mmol/l	11.39 (S.D. 4.9) (n=2)	11.14 (S.D. 0.41) (n=2)

S.D., standard deviation.

components were similar in wild-type and AChE −/− mice, thus giving no indication of malnourishment.

3.1.10. Breeding

The AChE knockout colony is maintained by breeding heterozygote males and females. We keep 50 female and 20 male AChE +/− breeders. The female AChE +/− breeders are 56 days to 1.5 years old, while the male breeders are 56 days to 2 years old. The average 129Sv female produces six litters in her lifetime, ranging up to eight. The litter sizes range from three to 12, and average 6.5; 15% of living newborns are AChE −/−. The females are bred approximately once every 2 months. This protocol yields 40–50 AChE −/− mice every 2 months.

3.2. Behavior

3.2.1. Home cage behavior

Normal mice select one remote area of their cage for defecation and urination. They avoid this area. By contrast, AChE −/− mice defecate and urinate in their nest. This unusual behavior suggests that higher brain function in AChE −/− mice is impaired, as in dementia, or is undeveloped as in neonates.

The function of the paper towel that lines the plastic box is to absorb urine. We started lining their houses with paper towels after we noticed that AChE −/− mice were shredding moist paper towels and stuffing the shreds into the top and sides of their plastic box. This activity decreased after we lined their houses.

AChE −/− mice, like normal mice, are sociable. Up to eight mice in a hamster cage crowd inside one plastic box. Each hamster cage has two plastic boxes. The group of mice moves to the second box after the paper towels in the first house are soaked with urine.

3.2.2. Vocalization

About 20–25% of AChE −/− mice make a bird-like chattering sound when there is a disruption in the environment, for example when the paper towels are being replaced in their cage. They do not chatter when they are nesting. Chattering begins around the time of weaning, but rarely at an earlier age. Chattering is not necessarily accompanied by signs of illness, though it is a sign of stress. Normal mice do not vocalize unless they are in distress, and then the sound they emit is a squeak. AChE −/− mice squeak under exceptional circumstances, for example when they are injected with a drug. AChE −/− mice make a third type of sound, a sharp chirp when they appear to suffer chronic pain.

3.2.3. Grip strength

Grip strength was measured by counting the seconds a mouse could hold on to an inverted screen before falling off. Fig. 5 shows that on postnatal day 11, all mice had poor grip strength, regardless of genotype. By day 17, the

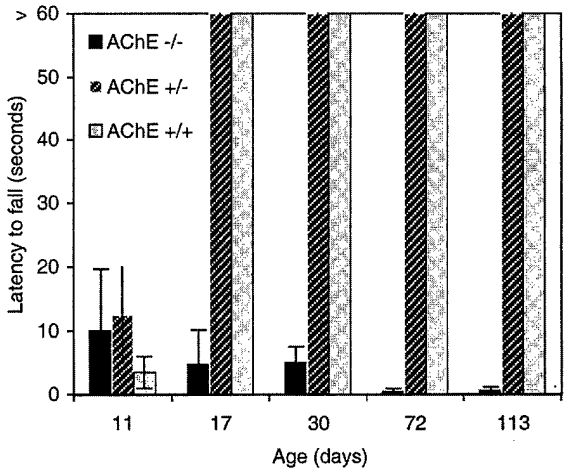

Fig. 5. Grip strength measured on an inverted screen. AChE +/+ (2 female+1 male), AChE +/− (2 females+1 male), AChE −/− (7 females+7 male) for the various age groups. Error bars show standard deviation.

AChE +/+ and +/− mice had acquired normal grip strength. However, AChE −/− mice never demonstrated improvement in their grip strength. By postnatal day 113, AChE −/− mice were weaker than on day 11. These results demonstrate that the muscles required for grip are weak in AChE −/− mice.

3.2.4. Gait

The abnormal gait of AChE −/− mice is illustrated in Fig. 6 in the pattern of footprints. Wild-type mice leave a

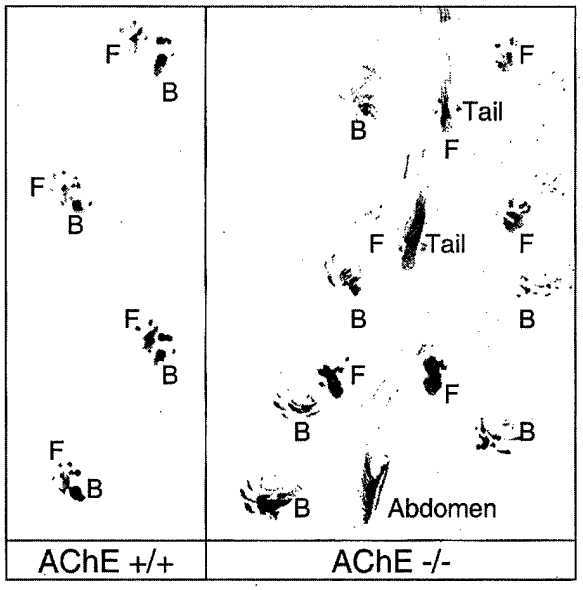

Fig. 6. Footprints. Front (F) and back (B) footprints of AChE +/+ mice fall close to each other in a tight pattern. By contrast, front and back footprints of AChE −/− mice are far apart. The foot splay is reflected in the large horizontal distance between prints. The abdomen and tail dragged through the paint. AChE +/+ and AChE −/− male mice of similar body weight (23 g) were selected for this experiment.

narrow trail of footprints, with the front and back prints falling nearly on top of each other. By contrast, AChE −/− mice leave a wide trail of footprints, due to the splayed position of their feet. AChE −/− mice drag their tail, shown in Fig. 6 as a streak of paint in the center of the pattern. Older AChE −/− mice walk with their abdomen very close to the ground. They have a hump in their back, giving them a hunched posture.

3.2.5. Locomotor activity

The AChE −/− mice appeared to be more sedentary than AChE +/+ mice. AChE −/− mice slept in their plastic box during simulated daylight hours, and came out only to eat. The juvenile play activity and territorial interactions common to wild-type mice were not noticed in AChE −/− mice. To quantitate these observations, locomotor activity was measured for two age groups. Fig. 7A compares locomotor activity of AChE +/+ and −/− mice, age 23–50 days. Both genotypes showed the pattern common to nocturnal animals, that is, highest activity at night, and lowest during the day. However, the activity level was lower in the AChE −/− mice for both the light and dark periods. Fig. 7B compares locomotor activity for mice, age 77–246 days. As in the younger group, mice were least active during the day light hours of 08:00 to 16:00 h and AChE −/− mice were less active than AChE +/+ mice. Comparison of Fig. 7A and B shows that younger animals were more active than older animals, but this effect of age was more obvious in wild-type mice.

3.2.6. Development of righting reflex

AChE −/− mice were slower to develop the righting reflex than their normal littermates (Fig. 8). By postnatal day 12, the littermates righted themselves within 1 s. However, AChE −/− mice did not acquire this level of righting ability until day 18.

3.2.7. Sexual dysfunction

AChE −/− mice are expected to be fertile because sperm have been found in the seminiferous tubule of males, and evidence of estrous has been found in females. Vaginal swabs yielded a variety of epithelial cells from cuboidal to cornified over a 4–6-day period. Normal female mice first enter estrous at 6–7 weeks of age, whereas AChE −/− females have estrous at 15–16 weeks of age, 9 weeks late. In normal male mice the testes descend at 4 weeks, whereas in AChE −/− males the testes descended late at 7–8 weeks. Despite these indicators of potential fertility, AChE −/− mice did not become pregnant. Male and female AChE −/− mice are housed together continuously during their life, but no mating behavior has been noticed. The males do not investigate female genitalia, do not mount, and do not groom females. It is possible that AChE −/− mice have a defect in the production of sex hormones or have stress-related infertility [22].

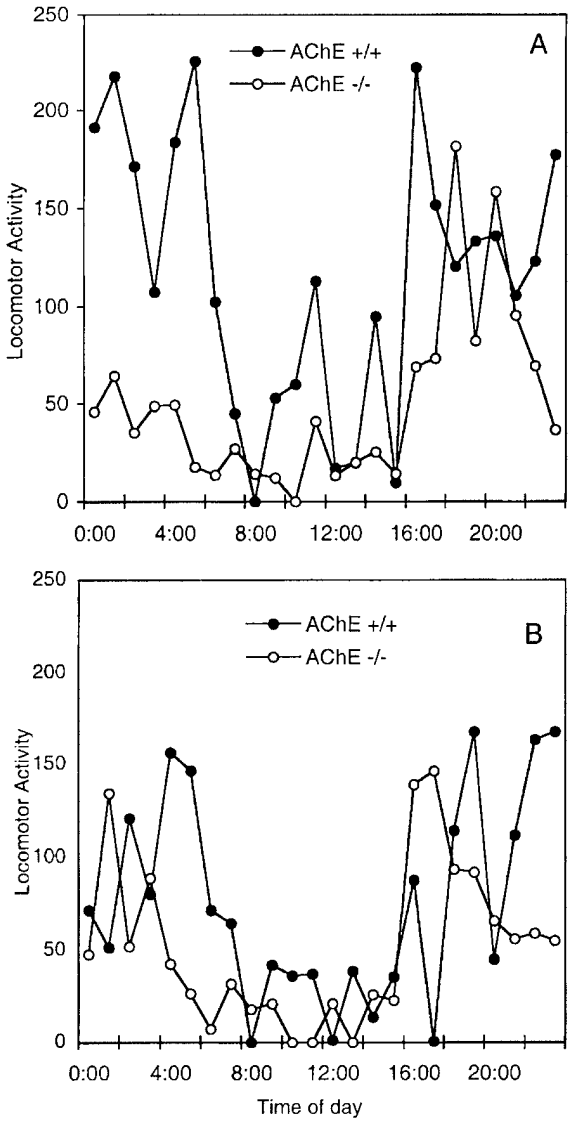

Fig. 7. Locomotor activity. Panel A, mice are 23–50 days old. Panel B, mice are 77–246 days old. $n=5$ in each group; all males. Lights are on for 12 h, from 06:00 in the morning to 18:00 h in the evening.

3.2.8. Smell

The failure of AChE −/− mice to mate suggested the possibility that they might have a defect in olfactory sensation. This possibility was tested by measuring their response to a novel food substance with a strong, attractive smell, namely peanut butter. Adult mice of each genotype and sex, 10 in each group, were tested. The time it took the mice to find and sniff the peanut butter ranged from 4 s to 4 min, and averaged 1.2±1.1 min for AChE +/+, 1.0±0.8 min for AChE +/−, and 1.0±1.0 min for AChE −/− mice. It was concluded that AChE −/− mice have normal olfactory sensation.

3.2.9. Stress

In response to prolonged restraint, AChE −/− mice showed signs typical of cholinergic toxicity. The eyes

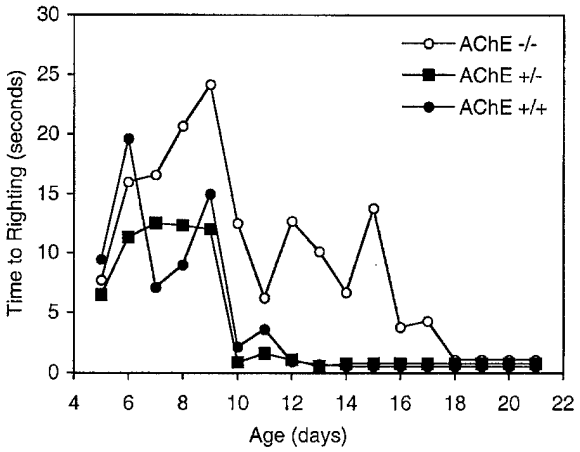

Fig. 8. Righting reflex as a function of age. The time from lying on the back to standing was measured for AChE −/− (23 female+25 male), AChE +/− (17 female+16 male), and AChE +/+ (7 female+7 male) mice. The mice had been fed Ensure Fiber.

clouded over with a thick mucus secretion, saliva wet the outside of the mouth and face, and body tremor intensified. If the mouse was not immediately released and allowed the comfort of a dark hiding place, it developed whole body seizures. AChE −/− mice often recovered from seizures. Alternatively, seizures progressed to tonic convulsions where the mouse body was propelled up to 30 cm above the lab bench. In most cases tonic convulsions resulted in death.

The daily handling of the mice to measure temperature and weight did not induce seizures. Seizures have been induced in some mice by sudden exposure to light, for example when the plastic box was lifted off the nest for its daily change of paper lining. On rare occasions a mouse has had seizures after it was placed into a novel environment.

AChE −/− mice were also susceptible to seizures and convulsions in their normal environment. Evidence that death had resulted from seizures and convulsions was the posture of the dead mouse: the front paws were curled under and the hind legs were extended. The stimulus that induced the convulsions is unknown, but it is clear that AChE −/− mice are sensitive to stress factors such as light, noise, and disruption of their normal environment.

3.2.10. Territorial aggressive behavior

Normal male mice housed together develop a social dominance hierarchy in which the dominant male defends the cage territory by attacking other males [5]. In these situations, dominant and submissive posturing is displayed. The AChE −/− male mice are not aggressive toward other male mice and do not demonstrate posturing behavior. However, there is a social dominance in AChE −/− mouse colonies, as demonstrated by barbering of whiskers, and loss of swatches of hair from the face, back and shoulders.

3.2.11. Defensive behavior

The normal instinct to bite when hurt or in distress is not seen in AChE −/− mice. Although these mice have teeth, they do not bite other mice or the handler even when provoked. They display almost no resistance when held.

3.2.12. Neurobehavioral screening

The literature overwhelmingly subscribes to the idea that the acute neurotoxicity of organophosphorus pesticides and other anti-cholinesterase agents results from the inhibition of AChE catalytic activity [18,24,29,33]. We reasoned that abnormal behaviors attributed to inhibition of AChE catalytic activity in wild-type mice, could be classified as behaviors due to loss of AChE catalytic activity in AChE −/− mice.

Behaviors that reflect neurotoxicity [20] were compared in adult AChE +/+, +/−, and −/− mice (Table 3). Mice with zero AChE activity were clearly different on several measures. All had whole body tremor, splayed feet, hunched back, and pinpoint pupils that did not respond to light. Older AChE −/− mice did not rear. Most AChE −/− mice did not move in the home cage unless provoked, but remained hidden inside their plastic box. When AChE −/− mice were held they did not struggle. They had little or no response when their tails were

Table 3
Neurobehavioral screening of 60–100-day-old mice of various AChE genotypes. There were 16 mice of both sexes in each genotype

Phenotype	AChE +/+	AChE +/−	AChE −/−
Mild whole body tremors	0/16	0/16	16/16
Hind legs splayed	0/16	0/16	16/16
Hunched back	0/16	0/16	16/16
Pupil response	16/16	16/16	0/16
Rearing (3–4 per min)	11/16	11/16	0/16
Unprovoked activity	15/16	16/16	1/16
Resistance to being restrained	16/16	15/16	2/16
Tail pinch response	15/16	15/16	2/16
Piloerection	0/16	0/16	3/16
Vocalization	0/16	0/16	4/16
Geotaxis	16/16	16/16	16/16
Righting reflex	16/16	16/16	16/16

pinched. AChE −/− mice that vocalized and displayed piloerection were also noticed to have bloating in the gastrointestinal tract. All adult AChE −/− mice had a normal response in the geotaxis test and all had the righting reflex. Mice with 50% of the normal AChE activity, AChE +/−, were indistinguishable from wild-type mice in behavioral tests. It was concluded that body tremor, pinpoint pupils, muscle weakness, and reduced pain response could be attributed to the absence of AChE catalytic activity in AChE −/− mice.

4. Discussion

4.1. Starvation due to weak muscles

In this report we have solved the problem of neonatal death in the AChE −/− mouse. Their average life expectancy has been extended to 100 days, from the 14 days in our first report [35]. The key to longer life was the realization that AChE −/− mice were starving to death under conditions in which their littermates thrived. The explanation for why only AChE −/− mice were starving is that AChE −/− mice have weak muscles. Their muscles are too weak to suckle enough milk from the dam's teats. When the fat content in the dam's diet was increased, the nullizygotes consumed more calories for the same effort, allowing them to survive longer. Hand feeding, early weaning, and regulating the ambient temperature also improved their survival.

Evidence for weak muscles in the AChE −/− mice includes their lack of grip strength, their abnormal posture and gait, their inability to eat solid food, inability to lift the head to drink from a suspended bottle, their low locomotor activity level, and lack of resistance to restraint. Weak muscles can be explained by malfunction of nerve impulse transmission at the neuromuscular junction, similar to that seen in endplate AChE deficient humans [10] and COLQ −/− mice [8].

4.2. Are adult AChE −/− mice malnourished?

On visual inspection, adult AChE −/− mice appear well nourished. They have a shiny, full coat of hair, and look plump. They have abundant fat in the abdominal cavity and in subcutaneous tissue. There are no abnormalities in the levels of blood components. The blood pressure and heart rate are normal. The mouse produces abundant fecal pellets and does not have diarrhea. Thus, the adult AChE −/− mouse does not have characteristics reported in semi-starvation and malnourishment [12,19].

However, the adult AChE −/− mouse also exhibits traits associated with under-nutrition including low body weight, low body temperature, limited locomotor activity, no grip strength, and sexual dysfunction. Some of these traits can be attributed directly to the absence of AChE and do not need a nutritional explanation. The absence of grip strength is due to abnormal muscle function, and the abnormal muscle function is explained by lack of AChE. The limited locomotor activity is a consequence of poor muscle function.

Other traits have no obvious connection to AChE, and may be an effect of under-nutrition. For example, the low body weight could be due to poor absorption of nutrients through the gastrointestinal tract. Nullizygotes consume more calories per gram body weight than wild-type mice, so poor feeding is not an explanation for low body weight. Hyper metabolism is another possible explanation for low body weight. If absence of AChE catalytic activity results in malabsorption of nutrients, then an explanation for how this could come about may be the following. Absence of AChE causes accumulation of excess acetylcholine. Excess acetylcholine causes a reduction in muscarinic receptors and this in turn decreases intestinal motility. The consequence of decreased intestinal motility might be malabsorption.

Another hypothetical scenario is based on the observation that AChE −/− mice, unlike wild-type mice, do not eat feces. Their lack of coprophagic behavior can be attributed to their weak muscles, which makes them incapable of chewing. The possibility exists that not being coprophagic results in an absence of normal microflora, and consequently under-nutrition. If the AChE −/− mice are undernourished then the degree of malnutrition is mild.

4.3. Role of AChE in development

The driving force for making the AChE knockout mouse and for prolonging its life to adulthood was the question of the nonclassical role of AChE during development [1–4,9,11,13–16,23,25,26,28,30–32]. The literature has abundant evidence that AChE has a second function, independent of its catalytic activity, and that this second, nonenzymatic role is most pronounced during development. Reviews on this topic are by Bigbee et al. [3] and Soreq and Seidman [31]. A summary of some of the evidence follows. AChE is specifically expressed in those areas of the rat thalamus and at those times when axons are projecting to the cortex. Once the nerves have grown to their destination in the cortex, AChE activity is extinguished. It has been hypothesized that AChE is a morphogen, guiding the growth of axons to those areas of the cortex involved with hearing, vision, and sensory response [25,26]. Studies in cultured neuronal cells have shown that binding of monoclonal antibody to AChE reduces neurite outgrowth [3], suggesting a morphogenic role for AChE. Inhibition of AChE activity in neuronal cultures has no effect on neurite outgrowth if the inhibitor binds at the bottom of the active site gorge, for example when the inhibitor is diisopropylfluorophosphate [3,30]. However,

an inhibitor that binds near the surface of the AChE protein, at the peripheral site, inhibits neurite outgrowth [3,15,23,28,32]. These results as well as the high sequence homology of AChE and cell adhesion proteins, have led to the hypothesis that the neuritogenic ability of AChE is mediated through a cell adhesion mechanism.

The most compelling evidence for a nonclassical function for AChE comes from studies of mutant zebrafish [2]. Behra et al. showed that AChE catalytic activity is required for neurite outgrowth in the zebrafish and that the structure of muscle fibers and of the neuromuscular junction was perturbed in zebrafish that had AChE protein but no AChE enzyme activity. Misrouting of primary motor neurons was attributed to accumulation of excess acetylcholine. Mutant zebrafish developed abnormally, had impaired motility, and died at early larval stages.

It was hoped that a mouse with no AChE protein would have obvious physical defects, for example abnormal brain structure, and would thus support the idea that AChE has a function in axon guidance during development. However, sections of brain olfactory mucosa, retina, cochlea, and striatum examined under light microscopy showed no structural abnormalities [35]. To date no axon tracing studies have been performed so it is unknown whether axon projections to the visual, auditory, and sensory cortex are normal. Another approach to this question is to test the ability of the AChE −/− mouse to see, hear, and sense. Detailed studies evaluating these senses are not complete, however AChE −/− mice startle from loud noise and bright light. When the prototypic neural cell adhesion molecule, N-CAM, was knocked out of the mouse, the phenotype of the knockout was mild [6]. It would not be surprising therefore, if the morphogenic function of AChE were compensated by other proteins in the mouse.

The AChE −/− mouse is characterized by postnatal developmental delay. Though the mouse eventually grows to the size of an adult, it never behaves like an adult mouse. It does not learn to defecate and urinate outside the nest, and it does not become sexually mature. The phenotype of the adult AChE −/− mouse provides evidence that AChE has a role in timely physical and central nervous system development. The fact that mice can live to adulthood in the complete absence of AChE enzyme and AChE protein shows that the functions of AChE are compensated. It has been speculated that the related enzyme, butyrylcholinesterase, compensates for the absence of AChE catalytic activity in nerve synapses [2,17,21].

4.4. The AChE −/− mouse as a model for intoxication by anti-cholinesterases

AChE is thought to be the physiologically important target of organophosphorus pesticides, chemical nerve agents, and anti-cholinesterase pharmaceuticals [18,24,29,33]. Acute inhibition of AChE to levels below 90% of normal leads to death. AChE knockout mice with zero AChE enzyme activity are alive, suggesting that adaptation to the absence of AChE occurred during development.

Does the phenotype of the AChE −/− mouse resemble that of the wild-type mouse poisoned with anticholinesterase agent? Wild-type mice poisoned with an organophosphorus nerve agent [7] have characteristics in common with untreated AChE knockout mice. The common characteristics are loss of AChE catalytic activity, the presence of body tremor, muscle weakness, susceptibility to seizures, reduced pain response, and pinpoint pupils. When stressed, AChE −/− mice salivate excessively and secrete a mucus covering on the eyes. Salivation and lacrimation are typical signs of anticholinesterase toxicity. We conclude that the AChE −/− mouse has many of the characteristics of the anticholinesterase intoxicated mouse, and will be useful as a model for gene and protein therapy studies aimed at treating toxicity.

The heterozygote AChE +/− mouse has 50% of the normal AChE activity [17]. Despite this deficiency, the mouse appears to be normal. One characteristic that distinguishes it from wild-type mice is a greater sensitivity to the toxic effects of organophosphorus poisons [7,35]. This feature is the basis for our prediction that humans partially deficient in AChE will be identified in the future, and that their health may be compromised by low doses of organophosphorus pesticides that have no ill effects on the general population.

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CRYSTAL STRUCTURE OF RECOMBINANT HUMAN BUTYRYLCHOLINESTERASE : NEW INSIGHTS INTO THE CATALYTIC MECHANISMS OF CHOLINESTERASES

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Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are two related enzymes with different substrate and inhibitor specificities. BChE hydrolyzes a large variety of ester-containing drugs such as cocaine and scavenges organophosphorous and carbamate toxic esters. Mutants of BuChE capable of hydrolyzing organophosphates have been designed. However, their activity needs to be improved to be of operational interest for prophylaxis and/or treatment of nerve agent poisoning, and decontamination purposes.

Unlike AChE, no X-ray structure of BChE is known, mainly because of the high glycosylation content of natural BChE, preventing crystal growth. Therefore, most structure studies of BChE, have relied on homology models built from the *Torpedo californica* AChE structure. Although these models have been instrumental in understanding some aspects of the AChE and BChE specificity differences, they are not accurate enough as templates for the rational design of mutants with particular catalytic features.

We recently crystallized a recombinant monomeric low-glycosylated form of human butyrylcholinesterase. The structure was solved at 2.0 Å resolution by molecular replacement using the *Torpedo californica* AChE structure as a starting model. Both enzyme structures are very similar. However, the active site of BChE presents an unexpected feature which may change the current interpretation of the molecular mechanisms of cholinesterases.

INTRINSIC TRYPTOPHAN FLUORESCENCE OF CHOLINESTERASES: DIRECT, NON-PERTURBING MONITORING OF ENZYME-LIGAND INTERACTIONS

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Intrinsic fluorescence of acetylcholinesterases (AChE, EC 3.1.1.7) and butyrylcholinesterases (BuChE, EC 3.1.1.8) was investigated to monitor enzyme-ligand interactions. The 10-13 tryptophans of cholinesterases emit fluorescence in 330 - 340 nm range upon excitation with UV light. Stern-Volmer analysis of collisional quenching of mouse AChE fluorescence by NaI, in the absence and presence of inhibitory peptide fasciculin 2, indicates that tryptophans of the AChE active center gorge contribute disproportionally to the overall fluorescence. Binding of non-fluorescent ligands, nonabsorbing in 330-340 nm range and incapable of resonance energy transfer, such as decamethonium, BW286c51, edrophonium, ethopropazine, acetylcholine and choline, quench AChE and BuChE fluorescence. However, binding of fasciculin 2 and carbamoylation of the active serine increase mouse AChE fluorescence intensity about 15%, thus suggesting conformational change involving triptophanes in the interaction. The rate of the conformational change appears faster than equilibration of reversible enzyme-inhibitor complexes thus allowing monitoring of inhibitor association and dissociation reactions in the millisecond time frame. Use of catalytically inactive mouse AChE S203A mutant allows for direct titration of AChE with acetylcholine revealing two binding sites for the substrate, one with Kd of ~80 uM and the other one of ~30 mM. In addition, measurements of rates of quenching of intrinsic AChE fluorescence upon association of edrophonium with mouse wild-type and H447I mutant AChE demonstrated a pH dependence consistent with the protonation state of H447 of the catalytic triad. In conclusion, monitoring of intrinsic tryptophan fluorescence of AChEs and BuChEs, is a sensitive and non-perturbing method of studying interactions of these enzymes with variety of nonfluorescent ligands. (Supported by grants from the NIH and DAMDC).

SURPRISING FINDINGS FROM THE FUNCTIONAL ANALYSIS OF HUMAN ACETYLCHOLINESTERASE ADDUCTS OF ALZHEIMER'S DISEASE DRUGS

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Determination of the 3D-structure of acetylcholinesterase (AChE) of *Torpedo californica* over a decade ago and more recently that of human enzyme together with extensive targeted mutagenesis of the mammalian AChEs led to a fine mapping of the multiple *functional* subsites within the active center of the enzyme. Our library of single and multiple human AChE mutants defining the various subsites was used to kinetically analyze interactions with various AChE inhibitors including tacrine (Cognex), huperzine-A, rivastigmine (Excellon), physostigmine, pyridostigmine, E2020 (Aricept, Donepezil) and galanthamine which are considered or currently in use for the treatment of Alzheimer's disease (AD). Such functional analysis characterized the key domains within the active center that are essential for accommodation of these prototypic inhibitors. Furthermore it allowed defining major structural features of the individual inhibitors that determine affinity and specificity for the enzyme. Some important and unexpected interactions were revealed by the functional analysis that could have not been anticipated from the 3D-structure of inhibitor-AChE complexes. These findings emphasize the importance of complementing the structural data with functional characterization of biological target molecules. Thus it appears that screening of lead compounds with a library of human AChE mutants may be a very useful and cost effective way for structure-based design and development of new therapeutics for AD.

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UNFOLDING AND FOLDING OF *TORPEDO CALIFORNICA* ACETYLCHOLINESTERASE

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Chemical modification of Cys231 in *Tc*AChE by various sulfhydryl reagents results in its conversion to one of two principal states. One of these states, produced by disulfides and by alkylating agents, is a stable state which displays typical features of a partially unfolded molten globule (**MG**) state, based on CD, intrinsic fluorescence and ANS binding. The second state, produced by mercury derivatives and the natural thiosulfinate, allicin, which has spectroscopic characteristics very close to those of the native (**N**) state is metastable: at room temperature it converts spontaneously with a half-life of ~1.5 h to the **MG** state. We named this state quasi-native (**N***). Demodification of *Tc*AChE in the **N*** state by glutathione or cysteine causes rapid release of the bound reagent, and concomitant recovery of most of the enzymatic activity. In contrast, similar demodification of the **MG** enzyme produces no detectable recovery of enzymic activity. Transition to the **MG** state of **N** *Tc*AChE, as well as of enzyme in the **N*** state, is greatly accelerated in the presence of phosphatidylcholine liposomes. Introduction of osmolytes (glycerol, sucrose, tetramethyl *N*-oxide), as well as of certain divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+}) retards transition of both the **N** and **N*** states to the **MG** state. The mechanisms underlying transition of the **N** and **N*** states to the **MG** state both in the absence and presence of liposomes, as well as the stabilisation induced by chemical chaperons, will be discussed according to the following scheme: **N** $\Leftrightarrow$ **N*** $\Rightarrow$ **MG**

X-RAY STRUCTURE OF SOMAN-AGED HUMAN BUTYRYLCHOLINESTERASE

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Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) phosphylated by branched organophosphates (OP) undergo a dealkylation reaction ('aging'), leading to OP-enzyme conjugates that cannot be reactivated by oximes. Previous studies from our laboratory revealed that aged-BChE conjugates are more stable to high temperature and pressure than the native enzyme. The increase in stability was shown to be related to changes in the water structure network in the active site gorge and to the formation of a salt bridge between PO- and protonated His438. Such a bridge was observed in the crystal structure of soman-, sarin- and DFP-'aged' AChE. These x-ray structures also revealed that the reactivation of aged conjugates was prevented by the stabilizing effect of H-bonding between a P bound oxygen and the oxyanion hole, and by the 'drying' effect of the acyl-binding pocket, preventing the nucleophilic attack. To complete our understanding of the molecular mechanism of the aging reaction of phosphylated BChE, the X-ray structure of a engineered form of human BChE has been determined. The structure of the soman-BChE aged conjugates was solved to 2.4 Angstroem resolution and compared to the aged conjugates of Torpedo californica AChE. Because the aged conjugate has no activity, we used this feature to determine the binding location of butyrylthiocholine in the active site of the phosphylated enzyme. The structure of the ternary complex aged BChE/butyrylthiocholine was solved to 2.3 Angstroem resolution. This later structure was aimed to provide new information on the peripheral site of BChE and the related phenomenon of substrate activation.

STUDIES ON DYNAMICAL TRANSITIONS IN CHOLINESTERASES

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Acetylcholinesterase (AChE) hydrolyses the neurotransmitter, acetylcholine, very rapidly, as required for termination of impulse transmission at cholinergic synapses. Its 3D structure reveals a deeply buried active site accessed by a narrow gorge [1]. This unanticipated structural characteristic raises cogent questions concerning traffic of substrates and products to and from the active site. It is obvious that substantial 'breathing' motions of the protein are required for penetration of substrates to the active site via the gorge and for clearance of products via routes as yet undetermined. It is, therefore, important to characterise the dynamics of the enzyme on various time scales. We have chosen incoherent elastic neutron scattering (IENS) [2] to investigate global atomic dynamics of both hydrated Drosophila melanogaster AChE (DmAChE) and of DmAChE dried by lyophilization as a function of temperature, and compared them to corresponding samples of human butyrylcholinesterase, an enzyme structurally very similar to AChE whose biological function is still unkown. The atomic mean square displacements (MSD) obtained by this technique reveal a dynamical transition for both hydrated samples. Such a transition has been observed previously for myoglobin [3], as well as for bacteriorhodopsin [4], and has been associated with the onset of biological function. Surprisingly, MSDs of the dry samples exceed those of their hydrated counterparts in the intermediate temperature range. Future objectives include theoretical interpretation of these findings, as well as investigation by IENS of the influence of covalently and reversibly bound inhibitors on the dynamics of these enzymes. [1] Sussman J.L., Harel M., Frolow F., Oefner C., Goldman A., Toker L. & Silman I. (1991). Atomic structure of acetylcholinesterase from Torpedo californica: A prototypic acetylcholine-binding protein. Science 253, 872-879. [2] Zaccai, G. (2001). How Soft is a Protein? A Protein Dynamics Force Constant Measured by Neutron Scattering. Science 288, 1604-1607. [3] Doster W., Cusack S. & Petry W. (1989). Dynamical transition of myoglobin revealed by inelastic neutron scattering. Nature 337, 754-756. [4] Ferrand M., Dianoux A.J., Petry W. & Zaccai G. (1993). Thermal motions and function of bacteriorhodopsin in purple membranes : Effects of temperature and hydration studied by neutron scattering. Proc. Natl. Acad. Sci. USA 90, 9668-9672.

TETANIC FADE IS REVEALED BY BLOCKING PRESYNAPTIC NICOTINIC RECEPTORS CONTAINING ALFA4BETA2 AND ALFA3BETA2 SUBUNITS AFTER REDUCING THE SAFETY FACTOR OF NEUROMUSCULAR TRANSMISSION

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The effects of subtype specific nicotinic receptor (nAChR) antagonists were studied on nerve-evoked tetanic (50Hz, 5sec) ontractions and [3H] acetylcholine release from rat isolated hemidiaphragms. nAChR antagonists reduced tetanic peak tension with a rank potency order of alfa-bungarotoxin(BTX)>d-tubocurarine(TC) >> mecamylamine(Meca) > hexamethonium(Hex). Depression of tetanic peak tension by dihydro-beta-erythroidine(DHbE, 0.03-10 microM, an alfa4beta2 and alfa3beta2 antagonist), methyllycaconitine(MLA, 0.003-3 microM, an alfa7 antagonist) and alfa-conotoxin MII(CTX MII, 1-300 nM, a selective alfa3beta2 antagonist), did not exceed 30%. TC (0.1-0.7 microM), Meca (0.1-300 microM) and Hex (0.03-3 mM) induced both tetanic fade and peak tension depression. With DHbE (0.03-10 microM) and CTX MII (1-300 nM), tetanic fade was only evident after decreasing the safety factor of neuromuscular transmission (MgCl2, 6-7 mM). Neither BTX (3-100 nM) nor MLA (0.003-3 microM) produced tetanic fading. The antagonist rank potency order to reduce (50%) evoked [3H]-ACh release from motor nerve terminals, was CTX MII(100 nM)>TC(1 microM), DHbE(1 microM)>Hex(1 mM); BTX (300 nM) failed to affect ACh release. The results suggest that neuromuscular block is linked to the activation of BTX-sensitive nAChRs containing alfa1-subunits, whereas preferential blockade of neuronal alfa4beta2- and alfa3beta2-containing receptors cause tetanic fade by reducing nicotinic autofacilitation.

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MOLECULAR CHARACTERISATION OF ACETYLCHOLINESTERASE FROM THE PEACH-POTATO APHID MYZUS PERSICAE(SULZ.)

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The peach-potato aphid, Myzus persicae, is an extremely important pest that occurs worldwide causing direct feeding damage to many field and glasshouse crops. It is, more importantly, a major vector of many plant viruses including virus yellows in sugarbeet. A modified acetylcholinesterase (AChE) that confers specific insensitivity to pirimicarb and triazamate has been previously reported in Myzus persicae. In order to determine the genetic basis of this insensitivity, the AChE gene was amplified from both sensitive and insensitive forms using RT-PCR. However, no mutations were identified which could account for this insensitivity. The presence of a second gene was thus proposed. Alternative degenerate primers were designed based on published ace sequences from both vertebrate and invertebrate organisms. This led to the amplification of a partial sequence that differed from Myzus Ace1 sequence. Gene specific primers have been designed based on this sequence to characterise any mutations found in an insensitive form of the enzyme. Additionally, AChE has also been purified using affinity chromatography. The results of direct sequencing of this AChE protein is compared with amplified sequence from PCR to verify the second Ace sequence.

SITE-SPECIFIC ANALYSIS OF GLYCAN STRUCTURES ON PLASMA-DERIVED HUMAN (Hu) AND HORSE (Eq) BUTYRYLCHOLINESTERASES (BChE)

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The successful use of plasma-derived cholinesterases as pretreatment drugs for organophosphate toxicity, stems from their long mean retention times. The relatively high stability of these enzymes in circulation may be attributed to the number and structures of their carbohydrate residues. Therefore, the site-specific carbohydrate (CHO) structures of several soluble BchEs were determined. Purified proteins were fragmented by enzymatic or chemical means. Glycopeptides were purified by reverse-phase HPLC and identified by N-terminal sequencing. Asparagine-linked glycans were released by treatment with PNGase F. The free reducing ends were labeled with 8-aminonapthalene-1,3,6-trisulonate (Glyko, Inc.), and subjected to fluorophore-assisted carbohydrate electrophoresis analysis following sequential digestion with various glycosidases. Site-specific CHO structures were determined for 8/9 Hu and 3/8 Eq BChEs. For Hu BChE, the major structure consisted of an asialylo, galactosylated biantennary oligosaccharide without core fucosylation. Hu BChE site 8 (aa#481) and 9 (aa#486) were inseparable with the cleavage strategies employed, which precluded an absolute assignment of CHOs for these sites. Glycans of Eq BChE, were similar except that the sites displayed less microheterogeneity and the majority of structures were sialylated. While all the sites for Hu BChE displayed some degree of microheterogeneity, some sites were more heterogeneous; for example site 4 (aa# 241) had 25% oligo-mannose structures, while sites 5 (aa# 256) and 7 (aa# 455) consisted of 11% and 12% tri-galactose, tri-antennary structures, respectively. The notable lack of sialylation of Hu BChE CHOs was probably due to the prolonged storage of the glycopeptides. CHO structures for fewer Eq BChE sites were determined, but a direct comparison (Hu site 3 and Eq site 2) indicated a high degree of homology between them.

RECOVERY FROM DESENSITIZATION OF A NEURONAL NICOTINIC RECEPTOR

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We have shown (J. Pharm. Exptl. Ther. 289:656.1999) that skeletal muscle nicotinic acetylcholine receptors (nAChR) recover from desensitization depending on concentration and duration of exposure to agonist and its identity. Recovery after ACh exposure was significantly slower than that induced by nicotine (Nic) for all concentrations. Further, the muscle type nAChR displays a 'molecular memory' probably resulting from the conformational state that the receptor undergoes in response to agonist-specific desensitization.

Using whole cell patch voltage-clamping (EPC9) combined with rapid agonist delivery (Warner Instruments, Fast-Step), we examined responses to ACh and Nic in SHSY-5Y cells expressing the ganglionic, neuronal nAChR ($\alpha3\ \beta4$; $\alpha5\ \beta2$). Our data indicate that recovery from desensitization induced with ACh or Nic proceeds in a double exponential fashion except for the shortest durations of exposure we used (1 s or less) in which recovery kinetics were best fit with a single exponential. In all cases, recovery from desensitization proceeded considerably faster when ACh induced desensitization. Further, when ACh was used to desensitize and Nic to measure recovery, the kinetics of recovery were similar to those found when Nic alone was used to measure responses. Similarly, when Nic was used to desensitize and ACh to monitor recovery, kinetics were similar to those where cells were exposed to ACh alone.

We conclude that the neuronal receptor examined here behaves differently from the muscle type with respect to desensitization and recovery and that this difference is consistent with the known addictive effects of nicotine on neuronal cholinergic receptors.

MECHANISM AND STRUCTURAL REQUIREMENTS OF XANOMELINE WASH-RESISTANT BINDING TO M1 MUSCARINIC RECEPTORS

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Xanomeline is a novel agonist functionally selective for muscarinic M1 receptors. It is unusual in that it binds to receptors both in a reversible manner (with submicromolar affinity) and in a wash-resistant, quasi-irreversible manner (with micromolar affinity). We investigated the two modes of its binding on membranes from CHO cells expressing M1 muscarinic receptors, with the following conclusions:

(1) Reversible binding of xanomeline occurs at the classical binding site and is competitive with that of classical muscarinic ligands. (2) Stable binding (half-life of >24 h) is to an "exosite", distinct from the classical site. (3) Stable binding to the exosite is preceded by initial high-affinity binding, but an interaction of xanomeline with the classical binding site is not a prerequisite for its binding to the exosite. (4) Comparison of xanomeline with its analogues indicates that the wash-resistant binding of xanomeline depends on the presence of the O-hexyl arm in its molecule and that an at least five-carbon long O-alkyl chain is required for wash-resistant binding.

The length of the O-alkyl chain required and thermodynamic data suggest that the wash-resistant binding depends on the penetration of the O-alkyl chain of xanomeline between individual alpha-helices of the binding pocket and its direct interaction with lipids surrounding the receptor.

IS THE G-PROTEIN-COUPLED M2 MUSCARINIC RECEPTOR A VOLTAGE SENSOR?

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G-protein coupled receptors (GPCRs) are not considered to exhibit voltage sensitivity. Here, using Xenopus oocytes, we examined whether a classical GPCR, the muscarinic M2-receptor (m2R), is by itself a voltage sensor. Oocytes expressing m2R were voltage-clamped at -60 mV or at +40 mV, and the relationship between the G-protein-gated inwardly rectifying K+ channels (GIRK) response and acetylcholine (ACh), (a positively charged agonist) or oxotremorine (OXO), (an uncharged agonist), concentration was established at these two holding potentials. We found, for both agonists, that at +40 mV there is a shift to the right of the dose response curve. Direct binding experiments of [3H]-ACh to individual oocytes expressing m2R showed that the specific binding of [3H]-ACh was reduced by depolarization. These results suggest that the m2R senses changes in membrane potential.

EXPRESSION OF THE CHOLINERGIC GENE LOCUS IN THE TRACHEAL EPITHELIUM OF THE RAT

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Cholineacetyltransferase (ChAT) catalyzes the biosynthesis of aceylcholine (ACh) from acetyl-CoA and choline in the axoplasm. The vesicular acetylcholine transporter (VAChT) is responsible for the translocation of ACh into the interior of synaptic vesicles. The gene coding for ChAT contains the entire intronless sequence for VAChT within its first intron. Northern blot analyses have demonstrated mRNAs of different sizes in the central and peripheral nervous system of the rat. A ChAT mRNA (cChAT) of a single size (about 4 kb) was detected in brain and spinal cord. In peripheral tissues mRNAs of different sizes and a peripheral ChAT (pChAT) which seems to be a splice variant of cChAT could be detected. The occurence of ChAT in non-neuronal tissue is known, but still unknown is the molecular identity of ChAT in the respiratory epithelium. Thus, we amplified the whole coding region of the rat tracheal epithelial ChAT mRNA. By RT-PCR a 2071 bp fragment could be detected, which shares a 99% sequence identity with cChAT. The expression of VAChT could be demonstrated for the first time in rat tracheal epithelium by RT-PCR. Amplification of the whole coding region revealed a sequence identity of about 99 % to the VAChT known from cholinergic neurons. In the present study we demonstrate the simultaneous expression of ChAT and VAChT in rat tracheal epithelium. The obtained sequencing data strongly suggest that the ChAT and VAChT proteins of rat tracheal epithelium are identical to that known from central cholinergic neurons. *(SFB 547, project C2)*

MUSCARINIC RECEPTORS AND TRP-CHANNELS IN PRIMARY SENSORY NEURONS OF THE RAT

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In sensory neurones acetylcholine interacts with G-protein coupled muscarinic receptors (MR), thereby activating a variety of second messenger systems (IP3, DAG, PLC, PKC). TRP-channels are a family of Ca2+-permeable cation channels that are activated by depletion of intracellular Ca2+-stores or subsequent to the stimulation of PLC isoforms. In in vitro expression systems, MR are coupled via Gq/11 proteins to transient receptor potential (TRP) channels. Stimulation is followed by an increase in [Ca2+]i. Similarly, MR activation leads to an increase in [Ca2+]i in sensory dorsal root ganglion (DRG) neurons. Therefore, we investigated the presence and localisation of TRP-channels in DRG neurons at transcriptional (RT-PCR), translational (immunohistochemistry) and functional (Ca2+-imaging) level. Total RNA of lumbar DRG contained mRNA for five out of seven channel subtypes. Proteins for the TRP1-, 3- and 6-channel could be demonstrated in subpopulations of sensory neurons. Perikarya that expressed the marker of presumably nociceptive neurons, the vanilloid receptor-1, were also immunoreactive for the TRP1-, 3- and 6-channels. Activation of Gq/11-coupled MR using muscarine was followed by an increase in [Ca2+]i. The rise in [Ca2+]i was abolished in presence of Nickel/Cadmium or after depletion of intracellular Ca2+-stores by application of thapsigargin. This further suggests the involvement of TRP-channels in the MR-mediated Ca2+-signalling. *(SFB 547, Teilprojekt C2; SFB 353, Teilprojekt A10)*

DIVERSE MOLECULAR MECHANISMS UNDERLYING CONGENITAL MYASTHENIC SYNDROMES

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Mutations of muscle acetylcholine receptors cause defective neuromuscular transmission through a variety of molecular mechanisms. AChR deficiency is the most common of the congenital myasthenic syndromes (CMS). However, it is not always clear how mutations within the AChR epsilon-subunit coding regions affect mRNA and protein levels at the endplate. We found different homozygous mutations, located in the M3-M4 cytoplasmic loop, in four patients with typical AChR deficiency. Surface expression in HEK293 cells showed that each is a null mutation. Surprisingly, in situ hybridsation in biopsies showed normal expression of epsilon-subunit mRNA. Thus the mutant epsilon-subunit mRNA transcripts are neither up-regulated nor preferentially degraded. Moreover, there was no compensatory increase in gamma-subunit mRNA suggesting that the normal low level of the gamma-subunit in human muscle is sufficient for survival of patients with epsilon-subunit null alleles.

Other CMS are due to abnormal ion channel function. In one case, abnormal fetal development had resulted in multiple joint contractures (arthrogryposis) of the fingers at birth, as well as CMS presenting in infancy. Mutational screening revealed heteroallelic mutation within the AChR delta-subunit, d756ins2 and dE59K. d756ins2 is a null mutation, but both adult and fetal AChRs containing dE59K show abnormally short burst lengths, predicting a "fast channel" phenotype. Thus dE59K causes dysfunction of fetal as well as adult AChRs explaining the presence of joint contractures which result from reduced fetal movement. This is the first description of the association of AChR gene mutations and arthrogryposis, but any mutation that disrupts fetal AChR function could underlie additional cases.

ACETYLCHOLINESTERASE KNOCKOUT MICE HAVE INCREASED SENSITIVITY TO SCOPOLAMINE AND ATROPINE

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It is generally accepted that continued stimulation of cells with agonists results in a state of desensitization or downregulation.The purpose of this work was to test the hypothesis that AChE -/- mice have reduced levels of functional muscarinic receptors. The toxicity of the antimuscarinic drugs, scopolamine and atropine ,was tested in acetylcholinesterase wild-type (AChE +/+), heterozygous (AChE +/-) and nullizygous mice (AChE-/-). Mice were injected i.p. with scopolamine or atropine dissolved in saline. In the case of a lethal dose, mice died within 2-15 minutes. The LD50 for scopolamine was 100 mg/kg in AChE +/+, 180 mg/kg in AChE +/-, and 35 mg/kg in AChE -/- mice. The LD50 for atropine was 250 mg/kg in AChE +/+, 215 mg/kg in AChE +/-, and 96 mg/kg in AChE -/- mice. The higher sensitivity of AChE -/- mice to muscarinic antagonists is consistent with the interpretation that these mice have fewer muscarinic receptors. These results correlate with data obtained in our laboratory (Bin Li et al.) demonstrating that AChE -/- mice are less sensitive to the muscarinic agonists, oxotremorine and pilocarpine. Taken together, these results support the conclusion that AChE -/- mice have adapted to excess acetylcholine by downregulating muscarinic receptors.

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DOWNREGULATION OF MUSCARINIC RECEPTORS IN MICE DEFICIENT IN ACETYLCHOLINESTERASE

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This study examined how acetylcholinesterase (AChE) knockout mice have adapted to the absence of AChE. AChE hydrolyzes acetylcholine to terminate cholinergic neurotransmission. Overstimulation of cholinergic receptors by excess acetylcholine is lethal However, AChE-/- mice live to adulthood. The hypothesis was tested that adaptation occurred through downregulation of cholinergic receptors. Muscarinic receptors were investigated by treating mice with muscarinic receptor agonists, pilocarpine and oxotremorine (OXO). In response to 200 mg/kg pilocarpine i.p., 10/10 AChE+/+, 5/10 AChE+/-, and 0/6 AChE-/- mice had seizures. Results indicated that AChE -/- mice have reduced numbers of functional muscarinic receptors. A second group of mice was treated with 1 mg/kg OXO s.c. OXO specifically stimulates muscarinic receptors inducing tremor, hypothermia, and salivation. 6/6 AChE+/+, 6/6 AChE+/-, and 0/6 AChE-/- mice had severe tremor, a drop in surface body temperature of 12

TARGETING OF THE HUMAN VESICULAR ACETYLCHOLINE TRANSPORTER TO CHOLINERGIC SUBDIVISIONS IN TRANSGENIC MICE

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The vesicular acetylcholine transporter (VAChT) and choline acetyltransferase (ChAT) are encoded within a single regulatory unit, called the cholinergic gene locus (CGL). To identify regions in the CGL that are important for the cell type-specific expression of VAChT in vivo, we tested fragments of the human CGL for their ability to confer correct expression of human VAChT to mouse cholinergic neurons. In our previous work (Neuroscience 96 (2000):707-22) we identified an 8.7 kb fragment from the human CGL that restricted expression of the embedded VAChT to somatomotor neurons in transgenic mice. In the present study, we report the generation and analyzation of two additional human CGL transgenes. The addition of 2.5 kb of downstream sequence to the existing transgene resulted in an extension of the expression of human VAChT to the cholinergic neurons of the medial habenular nucleus. The removal of 4.5 kb from the 5`-end of this construct completely abolished human VAChT expression in mice. Our data provide strong evidence for a mosaic model for CGL regulation in separate subdivisions of the mammalian cholinergic nervous system.

THE DIURNAL ACTIVITY OF ACETYLCHOLINESTERASE INHIBITORS

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Objective: Acetylcholinesterase inhibitors (AChEI), particularly donepezil, can induce sleep disorders in Alzheimer's disease patients. It is believed that acetylcholinesterase (AChE) displays diurnal variation, and that this circadian phenomenon may be linked to AChEI-induced sleep disturbances. We examined the potential effects of AChEI on the diurnal activity of AChE. Methods: A literature search was conducted to identify studies investigating the sleep-activity cycle of patients taking AChEI. Results: Published evidence suggests that AChE activity follows a circadian pattern. Drugs such as donepezil, which exhibit highly potent AChEI activity, seem to interrupt this natural diurnal activity. As donepezil has a long half-life (average, 70 hours), it remains in the body for long periods of time. Thus, even if given in the morning, donepezil concentrations can still remain high enough to create daytime acetylcholine levels at night, interrupting sleep. Donepezil may override the circadian rhythm of the cholinergic system, disturbing the sleep-activity cycle and resulting in high incidences of insomnia and other sleep-related events, and raising hypnotic medication use. Galantamine (Reminyl(R)) has a half-life of 6-8 hours, is a less potent AChEI than donepezil, and allosterically modulates nicotinic acetylcholine receptors. Unlike donepezil, galantamine appears not to perturb the diurnal activity of AChE. This could explain the lower incidence of sleep disturbances and concomitant hypnotic use with galantamine. Conclusion: While donepezil may override the natural diurnal activity of AChE resulting in increased sleep disturbance, galantamine appears not to perturb this activity, giving a lower incidence of insomnia and sleep-related disorders.

THE MUSCARINIC M1 RECEPTOR AS A THERAPEUTIC TARGET FOR COGNITIVE DEFICITS: PRECLINICAL PHARMACOLOGY AND KNOCKOUT MOUSE STUDIES

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Muscarinic M1 receptors are localised to brain regions involved in learning and memory and have been implicated in the regulation of cognitive processes at the behavioural and molecular level. However, the specific muscarinic receptor subtypes involved in cognitive processing have not been definitively identified. With the availability of muscarinic receptor knockout mice, the effects of various receptor subtypes on learning and memory can be examined. In the present studies, spatial learning and memory as well as open field activity were assessed in M1 knockout (KO) mice and age-matched wild type (WT) controls. Significant deficits were found for M1-KO mice compared to controls in task completion time, reference memory and total errors. M1-KO mice had a significantly greater rate of ambulation on Days 1, 3 and 14. Results indicate that M1-KO mice exhibit impaired learning and memory in a spatial discrimination task and that this effect was independent of differences in activity level which were short-lived with extended monitoring. Within the hippocampus, the M1 receptor, but not the functionally similar M3 receptor, stimulated G protein activation and calcium mobilizing signaling pathways. Broader studies with mice bearing a genetic deletion of each of the 5 muscarinic receptor subtypes suggests that muscarinic receptor-mediated activities, such as tremor, salivation, hypothermia, heart rate, and smooth muscle function are regulated predominantly through the M2 and M3 receptors. Taken together, these studies indicate that the M1 receptor is a likely target for the development of therapeutics for the treatment of cognitive deficits.

RESCUE OF THE ACETYLCHOLINESTERASE KNOCKOUT MOUSE BY FEEDING A LIQUID DIET; PHENOTYPE OF THE ADULT ACETYLCHOLINESTERASE DEFICIENT MOUSE

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Acetylcholinesterase (AChE, EC3.1.1.7) functions in nerve impulse transmission, and possibly as a cell adhesion factor during neurite outgrowth. These functions predicted that a mouse with zero AChE activity would be unable to live. It was a surprise to find that AChE -/- mice were born alive and survived an average of 14 days. The emaciated appearance of AChE -/- mice suggested an inability to obtain sufficient nutrition and experiments were undertaken to increase caloric intake. Pregnant and lactating dams (+/-) were fed 11% high fat chow supplemented with liquid Ensure,. AChE -/- pups were weaned early, on day 15, and fed liquid Ensure. Although nullizygous animals showed slow but steady weight gain with survival over 1 year (average 100 days), they remained small at all ages compared to littermates. They demonstrated delays in temperature regulation (day 22 vs 15), eye opening (day 13 vs 12), righting reflex (day 18 vs 12), descent of testes (week 7-8 vs 4), and estrous (week 9 vs 6-7). Significant physical findings in adult AChE -/- mice included body tremors, abnormal gait and posture, absent grip strength, inability to eat solid food, pinpoint pupils, decreased pain response, vocalization, and early death caused by seizures or gastrointestinal tract ileus. Behavioral deficits included urination and defecation in the nest, lack of aggression, reduced pain perception, and sexual dysfunction. These findings support the classical role for AChE in nerve impulse conduction and further suggest that AChE is essential for timely physical development and higher brain function.

ROLE OF MUSCARINIC RECEPTORS IN THE ACTIVATION OF THE SUBICULO-ACCUMBENS PROJECTION

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The nucleus accumbens receives limbic inputs from a number of brain regions, including the ventral subiculum. Activation of this projection, following microinjection of N-ethyl-D-aspartate (NMDA) or carbachol increases locomotor activity. Using in vivo microdialysis, ventral subiculum application of NMDA increases levels of dopamine in the nucleus accumbens. Experiments were conducted to ascertain, in the nucleus accumbens, the neurochemical consequences of carbachol administration using microdialysis, and to explore the cholinergic receptor subtype(s) involved in any evoked response. In anaesthetised rats, ventral subiculum administration of carbachol increased dopamine levels in the nucleus accumbens. Administration of nicotine or the alpha-7 nicotinic acetylcholine receptor agonist, AR-R17779 failed to evoke a response. An involvement of muscarinic receptors was suggested from the significant reduction in response to carbachol following co-administration with atropine. Sensitivity of the subiculo-accumbens projection to muscarinic cholinergic receptor stimulation was confirmed by a significant increase in nucleus accumbens dopamine following ventral subiculum administration of the broad-spectrum muscarinic agonist oxotremorine M. In further studies utilising subtype selective agonists, xanomeline (M1 and M4 preferring agonist) failed to increase dopamine. However, ([5R-[exo]-6-[butylthio]-1,2,5-thiadiazol-3-yl]-1-azabicyclo[3.2.1])octane (BuTAC - M2 and M4 partial agonist, and M1, M3 and M5 antagonist) evoked a significant response. These data show that the subiculo-accumbens projection is sensitive to muscarinic receptor stimulation, and suggests an involvement of the M2 receptor subtype.

BRAIN PENETRATION AND BEHAVIOURAL PROPERTIES OF A POTENT ALPHA 7 NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST IN THE RAT

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The homomeric alpha-7 nicotinic receptor is the second most abundant nicotinic receptor in the brain and has been implicated in a number of psychiatric and neurological disorders. We have evaluated PSAB-OFP ((R)-(+)-5'-phenylspiro[1-azabicyclo[2.2.2]octane-3,3'(3'H)-furo[2,3-b]pyridine], Phillips et al., Astra Arcus USA, patent WO99/03859 a potent alpha-7 agonist in a battery of behavioural assays in the rat. Initial studies confirmed that after systemic administration (5 mg/kg s.c.) the Cmax in rat brain was 5.8 microM with a T1/2 of 1.3 hr. We then went on to evaluate the effects in the rat of PSAB-OFP in locomotor activity, pre-pulse inhibition and on performance in the radial and Morris mazes. In addition the compound was examined against hyperalgesia induced by formalin and carrageenan and in 6-hydroxydopamine lesioned rats. Results indicated that PSAB-OPF decreased spontaneous locomotor activity 20-40 min after injection (20 % decrease at 10 mg/kg), but failed to alter stimulant-induced activity, pre-pulse inhibition or cognitive performance in either the Morris water maze or 8-arm radial maze. The compound was also inactive in hyperalgesia models and had no functional or neuroprotective actions in the 6-OHDA model. PSAB-OFP was thus inactive in a wide range of behavioural assays. It is not clear whether this reflects a relatively unimportant role for the alpha-7 receptor, insufficient receptor exposure to the compound and/or rapid receptor desensitisation. The 5-HT3 agonist cross-reactivity of this molecule also makes it difficult to make concrete conclusions on the role of alpha-7 receptors in these in vivo tests.

FINE-TUNING MODULATION OF NEURONAL MUSCARINIC M1 (FACILITATORY) AND M2 (INHIBITORY) RECEPTORS ACTIVATION BY ADENOSINE AT THE RAT NEUROMUSCULAR JUNCTION

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The crosstalk between adenosine and muscarinic autoreceptors regulating evoked [3H]-acetylcholine ([3H]-ACh) release was investigated on rat phrenic nerve-hemidiaphragms. Motor nerve terminals possess facilitatory M1 and inhibitory M2 autoreceptors. Dicyclomine (3 nM-10 microM) caused a biphasic (inhibitory/facilitatory) effect, indicating that M1-facilitation prevails during 5 Hz-trains. Co-activation of M2 receptors was partially attenuated, since pirenzepine (1 nM, an M1-antagonist) significantly enhanced inhibition by oxotremorine (10 microM). CGS 21680C (2 nM), an A2A-adenosine receptor agonist, (1) potentiated oxotremorine (10 microM) inhibition, and (2) shifted McN-A-343 (3 microM)-facilitation into a small inhibitory effect. Conversely, the A1-receptor agonist, R-PIA (100 nM), reduced the inhibitory effect of oxotremorine (10 microM), without changing facilitation by McN-A-343 (3 microM). Synergism between A2A- and M2-receptors is regulated by a reciprocal interaction with M1 receptors that can be prevented by pirenzepine (1 nM). During 50 Hz-bursts, facilitation (M1) of [3H]-ACh release by McN-A-343 (3 microM) disappeared, while M2 inhibition became predominant. This muscarinic shift results from the interplay with A2A-receptors, because it was prevented by the A2A antagonist, ZM 241385 (10 nM). Thus, when muscarinic M1 facilitation is fully operative, inhibition of ACh release is mediated by adenosine A1 receptors. During high frequency bursts, tonic activation of A2A receptors promotes M2-autoinhibition by braking M1 receptor counteraction.

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IDENTIFICATION OF SIGNALING PROTEINS DOWNSTREAM OF THE TYROSINE KINASE MUSK IN CLUSTERING OF ACTEYLCHOLINE RECEPTORS

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During development of the neuromuscular junction, nerve-released agrin causes clustering of acetylcholine receptors (AChRs) in the muscle membrane. The muscle specific kinase (MuSK) is part of the agrin receptor complex and undergoes agrin-induced dimerization and autophosphorylation, thereby starting a signaling pathway that eventually drives AChR phosphorylation and clustering. We observed previously that treatment of C2C12 myotubes with the kinase inhibitor staurosporine prevents aggregation of AChRs but leaves MuSK active, implying the activity of other kinases downstream of MuSK. To analyse possible interaction partners and/or substrates of MuSK, we immunoprecipitated MuSK and identified associated phosphorylated proteins by phosphotyrosine immunoblotting. One major band running at about 60 kDa was specifically coprecipitated by MuSK and phosphorylated on tyrosine in response to agrin treatment. As Src-family kinases bind to the AChR and are activated by agrin, we investigated the possibility that these kinases associate with MuSK and are phosphorylated due to agrin. In myotubes derived from mice lacking both Src and Fyn, no change in the phosphorylation extent of the MuSK-associated protein was observed. Staurosporine and the specific Src-family kinase inhibitors PP1 and CGP77675 did not affect the phosphorylation degree of the protein. Instead, it was reduced by treatment with herbimycin, in parallel with the reduction of MuSK phosphorylation. Finally, in myotubes lacking rapsyn, phosphorylation of the MuSK-associated protein was still observed. Thus, so far, the identification of the MuSK-associated protein as a member of the Src-family could not be confirmed, and we presently consider other muscle proteins of 60 kDa.

DRAMATIC DEPLETION OF CELL SURFACE ACETYLCHOLINE MUSCARINIC RECEPTORS m2R DUE TO LIMITED DELIVERY FROM INTRACYTOPLASMIC STORES IN NEURONS OF ACETYLCHOLINESTERASE (ACHE) DEFICIENT MICE

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The abundance of G protein-coupled receptors (GPCR) located at the neuronal membrane depend on complex intraneuronal trafficking involving delivery of GPCRs from the cytoplasm to the membrane. We have studied here the effect of the chronic AChE deficiency in neurons of AChE gene KO mice on the subcellular distribution of the m2R, using immunohistochemistry at light and electron microscopic levels. 1)a) In AChE+/+ mice in vivo, m2R is abundant at the plasma membrane in striatum, hippocampus and cortex. b) In AChE-/- mice, m2R is almost absent at the membrane but is abundant in endoplasmic reticulum and Golgi complex. 2) Dynamic studies show that the balance between membrane and cytoplasmic m2R depends on the cholinergic influence: a) In AChE-/- mice, the blockade of muscarinic receptors restores m2R at the membrane. b) In AChE-/- mice in vitro(organotypic culture), when acetylcholine is produced by interneurons (striatum), m2R is located in the cytoplasm as in vivo. The supplementation of AChE-/- neurons with AChE provokes a translocation of m2R from the cytoplasm to the membrane. c) In vitro, when AChE-/- hippocampus is disconnected from its cholinergic afference, m2R is located at the membrane. When AChE-/- hippocampus is co-cultured with AChE-/- septum, its cholinergic afference, m2R keeps the cytoplasmic distribution seen in hippocampus in vivo. Our data suggest that the neurochemical environment may contribute to the control of abundance and availability of cell membrane GPCR, and consequently to the control of neuronal sensitivity to neurotransmitters, by regulating their delivery from intracytoplasmic stores to the membrane.

CHEMICAL MODIFICATION OF RECOMBINANT HUMAN ACETYLCHOLINESTERASE BY POLYETHYLENE GLYCOL GENERATES AN ENZYME WITH EXCEPTIONAL CIRCULATORY LONGEVITY

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One of the major obstacles to the fulfillment of the therapeutic potential of recombinant human acetylcholinesterase (rHuAChE) as a bioscavenger of organophosphates is its short circulatory residence. Post-translation-related factors such as sialylation level and subunit assembly were recently shown to determine the circulatory fate of AChE and demonstrated the ability to generate recombinant AChE with improved pharmacokinetic traits. Here we show that the pharmacokinetic performance of rHuAChE can be increased significantly by the controlled attachment of polyethylene glycol (PEG) side-chains to lysine residues. The increase in mean residence time (MRT) of the PEG-modified monomeric enzyme is linearly dependent, in the tested range, on the number of attached PEG molecules as well as on their size. It appears that even low level PEG-conjugation can overcome the deleterious effect of sub-optimal post-translation modifications such as under-sialylation. Attachment of as many as four PEG molecule to monomeric rHuAChE had minimal effect if any on either the catalytic activity or on the reactivity of the modified enzymes towards active center inhibitors such as edrophonium and diisopropylfluorophosphate (DFP) or to peripheral site ligands such as propidium, BW284C51 and even towards the bulky snake-venom toxin - fasciculin-II. At the highest tested ratio of attached PEG-20,000 to rHuAChE (4:1), an MRT of over 2100 minutes was attained (compared to MRT of 42 minutes for the non-modified enzyme), a value unmatched by any other known form of recombinant or native plasma derived AChE reported to date. This provides an important step toward the generation of a pharmaceutically efficient recombinant AChE-based bioscavenger for prophylactic treatment of organophosphate poisoning.

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HUPERZINE A AND DONEPEZIL ATTENUATE STAUROSPORINE-INDUCED APOPTOSIS IN RAT CORTICAL NEURONS VIA BCL-2 AND BAX REGULATION AND INHIBITION ON CASPASE-3

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Staurosporine treatment results in apoptotic cell death ad DNA fragmentation. bcl-2, bax and Caspase-3 are known to regulate the apoptotic cell death. This study sought to examine effects of huperzine A and donepezil on staurosporine-induced neuronal apoptosis and potential mechanisms in primary cultured rat cortical neurons. Treated with 0.5 micromolar staurosporine for 24 hours results in significant decrease in cell viability, alteration of neuronal morphology and DNA fragmentation. Pretreatment of the cells with huperzine A and donepezil for 2 hours prior to staurosproine exposure markedly elevated the cell survival at 0.1-10 micromolar concentration and reduced staurosporine-induced nuclei fragmentation at 1 micromolar concentration. 1 micromolar huperzine A and donepezil pretreatment also reduced the upregulation of pro-apoptotic gene bax, the downregulation of anti-apoptotic gene bcl-2 as well as activation of Caspase-3. Thus our results provide the first evidence that huperzine A and donepezil protect neurons against staurosporine-induced apoptosis via the upregulation of bcl-2 and downregulation of bax and inhibition on Caspase-3 activity.

Crystal structure of native and soman-aged human butyrylcholinesterase: a key step for engineering of catalytic scavengers against organophosphate poisoning

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Enzymes capable of degrading highly toxic organophosphate (OP) esters are emerging as safe and efficient decontamination tools. Stoichiometric and catalytic biological scavengers are also promising alternative medical countermeasures against poisoning by nerve agents. Engineered cholinesterase-based enzymes, having high OP-hydrolyzing activity, are potential candidates and could be used for pretreatment, decontamination of skin, mucosa and open wounds or for treatment as supplement to current therapy.

Starting from the modeled structure of human BChE (EC. 3.1.1.8), mutants capable of hydrolyzing OP were designed. Although successful results, the catalytic activity of the mutants was too low to be of operational interest. To improve the efficiency of such mutants and to clearly establish the molecular mechanism underlying their phosphotriesterase activity, detailed knowledge of the BChE structure was necessary. For this purpose, a recombinant monomeric human BChE suitable for X-Ray crystallography was designed and crystallized (1).

The structures of native and soman-"aged" human BChE were respectively solved to 2.0 Å and 2.4 Å resolution (2). Those structures provide new information on the molecular aspects of the irreversible inhibition (aging) of BChE by soman and on the OP hydrolase mechanism of mutants having phosphotriesterase activity. Moreover, the active site of the native enzyme was found to display an unexpected major feature which is of importance regarding the mechanisms of catalysis and inhibition of cholinesterases (3).

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Revisiting mechanisms of catalysis and inhibition of butyrylcholinesterase: prospects for research on medical counter-measures against poisoning by nerve agents

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Butyrylcholinesterase (BuChE, EC. 3.1.1.8) is closely related to acetylcholinesterase (AChE, EC.1.1.7). Unlike AChE, which plays a key role in the cholinergic transmission, so far the physiological function(s) of BuChE remain(s) unclear. However, BuChE is of toxicological and pharmacological importance. In particular, BuChE scavenges organophosphorous compounds and mutants of this enzyme have been designed for hydrolyzing nerve agents.

Recent crystallization (1) and resolution of the x-ray structure of human BuChE at 2 Å provided evidence that native BuChE is a 'dormant' enzyme (2). This pecularity was found to affect the kinetic behavior of the enzyme:

Like AChE (3), BuChE was found to slowly reach steady-state velocity with the neutral ester N-methylindoxyl acetate as a substrate. The kinetic mechanism of this hysteretic process was interpreted in terms of slow conformational change of the enzyme from an inactive form E to an active form E' that binds and hydrolyzes substrate (4). Hysteresis by peripheral site mutants (D70G, Y332A and D70G/Y332A) was similar to that of wild-type enzyme. In addition, hysteresis was shown for hydrolysis of positively charged substrates by wild-type enzyme, the D70H mutant and different active site mutants, e.g., A328C and G117H/E197Q. This clearly indicated that hysteresis is a general property of BuChE which does not involve the peripheral site. Lags in progress curves for substrate hydrolysis can be interpreted as the time needed to convert the population of dormant enzyme E to the operative form E', performing catalytic turnover at steady-state velocity.

Irreversible carbamylation of BuChE by the photolabile inhibitor, N-methyl-N-(2-nitrophenyl)carbamoyl chloride (MNPCC) (5) was found to follow biphasic kinetics which can be described by a sum of two first-order processes. Yet, x-ray diffraction data revealed that the active site serine is the sole target of MNPCC (6). This result supports the contention that BuChE is composed of two enzyme populations, MNPCC reacting slowly with the dormant form E and fast with the operative form E'.

Why is the acetylcholinesterase knockout mouse supersensitive to organophosphorus agent (OP) toxicity?

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The biochemical event that triggers convulsions and death after exposure to organophosphorus agents is inhibition of acetylcholinesterase. When AChE is inhibited, excess acetylcholine overstimulates acetylcholine receptors. This starts a cascade of neuronal excitation involving the glutamate receptors and excessive influx of calcium ions, that is responsible for the onset and maintenance of seizure activity (McDonough & Shih, 1997). Inhibition of AChE has been documented in thousands of papers, and is the gold standard for measuring exposure to OP. This understanding of the mechanism of OP toxicity has led to effective therapies against OP poisons. Administration of atropine to block muscarinic receptors, of 2-PAM to reactivate AChE, and of anticonvulsant drugs, is standard clinical practice for treatment of OP toxicity. In view of the overwhelming evidence for a critical role for AChE in OP toxicity, it was of interest to determine the response of a mouse that had no AChE. If AChE were the only important target of OP, then mice with no AChE were expected to be resistant to OP toxicity.
Strain 129Sv mice, differing only at the ACHE gene locus, were tested with four organophosphorus agents.

LD_{50} of organophosphorus toxicants in AChE deficient and wild-type mice.

AChE Genotype	AChE activity	DFP LD_{50}	Chlorpyrifos Oxon LD_{50}	VX LD_{50}	Iso-OMPA LD_{50}
AChE +/+	100%	> 2.5 mg/kg	3.5 mg/kg	24 µg/kg	350 mg/kg
AChE +/-	50%	2.5 mg/kg	2.5 mg/kg	17 µg/kg	350 mg/kg
AChE -/-	0 %	< 2.5 mg/kg	0.5 mg/kg	11 µg/kg	3 mg/kg

It was found that AChE -/- mice died at doses of diisopropylfluorophosphate (DFP), chlorpyrifos oxon, VX, and iso-OMPA that were not lethal to heterozygote AChE +/- and wild-type AChE +/+ mice. Furthermore, heterozygote AChE +/- mice, who have 50% of normal AChE activity, were more susceptible to OP toxicity than AChE +/+ mice. These results lead to the conclusion that AChE is not the only physiologically important target of acute OP toxicity.
We have not yet identified the targets of OP toxicity in AChE knockout mice. Two additional targets might be butyrylcholinesterase and N-acyl peptide hydrolase (Richards et al., 2000). In VX treated AChE +/+ and +/- animals, both BChE and AChE were inhibited about 50% (Duysen et al., 2001). In VX treated AChE -/- mice, BChE activity was inhibited 50%. By contrast, N-acyl peptide hydrolase was not significantly inhibited in VX lethalities. These results suggest that butyrylcholinesterase might be a physiologically important target for OP agents, though other unknown targets might also be involved.

CONTRACTILE, ELECTROPHYSIOLOGICAL AND MORPHOLOGICAL PROPERTIES OF DIAPHRAGM MUSCLE IN ACETYLCHOLINESTERASE KNOCKOUT MICE

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A mouse knockout mutant that is completely lacking acetylcholinesterase (AChE) has been produced and adaptations in skeletal muscle synapses present in the homozygous strain (AChE -/-) that allow survival were examined. Nerve-evoked contractions were studied *in vitro* in phrenic nerve-hemidiaphragm preparations from AChE -/- mice and their age and sex matched wild-type littermates. Twitch tensions elicited in diaphragms of AChE -/- mice by single supramaximal stimuli had 5-fold larger amplitudes, 2-fold slower rise-times, and 2-fold greater durations at ½ peak than those recorded from control wild-type mice. In AChE -/- mice, repetitive stimulation at frequencies of 20 and 50 Hz led to a gradual decline in amplitude, culminating in approximately a 30% loss of tension during the 500 msec stimulation period. Surprisingly, little decline of tension was observed during stimulation at 70 and 100 Hz, suggesting that the muscle was able to function at or near control levels in the physiological frequency range in spite of the complete absence of AChE. Tetanic summation was less in the AChE -/- mice, resulting in convergence of sustained tensions at 100 Hz (10 ± 3 grams) to those seen in wild-type controls (7 ± 2 grams). With additional increases in frequency (200-700 Hz), tensions exhibited marked tetanic fade. Tension-frequency profiles similar to those described above could be elicited in wild-type preparations after acute exposure to the selective AChE inhibitor BW 284C51 (1 μM) and 0.4 μM d-tubocurarine. These results suggest that the diaphragm muscle in the mutant mice may adapt to the loss of AChE by reducing the density of acetylcholine (ACh) receptors, leading to accelerated diffusion of ACh. This was confirmed by findings that diaphragms from AChE -/- mice had significantly lower ACh receptor densities as revealed by fluorescent bungarotoxin binding. Successful adaptation of diaphragm muscle from AChE -/- mice was also revealed by microelectrode recordings. These indicated that evoked endplate potentials (epps) and miniature endplate potentials (mepps) from AChE -/- mice had no significant alteration in amplitude and time course and that the quantal content of the epp was also unchanged in AChE -/- mice. Ultrastructural examination of diaphragm muscles from AChE -/- mice revealed smaller nerve terminals, irregular junctional folds and invasion of the nerve terminal by Schwann cells. Most of these appear to be adaptive in limiting the effect of excess AChE. A combination of reductions in ACh receptor density and morphological alterations appear to account for the successful adaptation of AChE -/- mice to survive an AChE-free environment.